This thesis is dedicated to my mother and father, for the years of guidance and support which has made it possible

IMMUNITY TO THE TAPEWORM

MESOCESTOIDES CORTI

(Cestoda: Cyclophyllidea)

by

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A thesis submitted for the degree of
Master of Science
at the
Department of Zoology,
Australian National University.

December 1977

PREFACE

In this thesis the immunity in mice to the tapeworm Mesocestoides corti is studied.

Assistance was given by Dr. C. Parish of the John Curtin School of Medical Research (JCSMR) with the cell separation of peritoneal cell suspensions. The plaque forming cell technique was demonstrated by Dr. A. Cunningham, (JCSMR). Thymus cell grafts in athymic mice were carried out by Dr. G. Mitchell of the Walter and Eliza Hall Institute.

Programs for the statistical tests, Bartlett's Chi-Square and ANOVA, were supplied by Dr. T. Marples and the statistical analysis using the Student-Newman-Keul's test was carried out by Dr. W.L. Nicholas.

Dr. A. Stewart helped with the preparation of liver digestions and with the counting of the parasite recovery when large numbers of mice were involved. Help with the histological preparations and photography of material was given by Mr. N. Call, and Dr. A. Stewart.

The surgical operations of mice for the production of burnt liver lesions were carried out with the help of Dr. P. Janseen.

All irradiations of mice and tetrathyridia were carried out at the C.S.I.R.O. (Canberra).

No part of this thesis has been submitted for a degree at any other university.

S. Pollacco.

S. Pollacco

ACKNOWLEDGEMENTS

I would like to take the opportunity to thank all those who have contributed in some way towards the work described in this thesis. In particular, thanks to my supervisor, Dr. W.L. Nicholas, for his help and encouragement throughout the project.

Sincere thanks to Dr. C. Parish and to Dr. A. Cunningham, both of the John Curtin School of Medical Research, for their advice and demonstration of immunological techniques. I would like to thank Dr. G. Mitchell of the Walter and Eliza Hall Institute for Medical Research for supplying me with inbred mice, especially the athymic mice, and also for carrying out thymus cell grafts in athymic mice.

I am grateful to Dr. A. Stewart and Mr. N. Call for their advice and help in the preparation of histological sections and Mr. I. Fox and Dr. A. Stewart for photography. I would also like to thank Dr. T. Marples for his advice on statistical analysis and for supplying me with statistical programs. Thanks also to Dr. P. Janseen for helping me with the surgical operations.

I would like to acknowledge the officer at C.S.I.R.O. for giving me the opportunity to use the ^{60}Co irradiation unit.

The award of an Australian National University Scholarship is gratefully acknowledged.

I am also indebted to my husband and my parents for their encouragement throughout.

S. Pollacco

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ABSTRACT

Mice, both of the outbred and inbred strains, can be experimentally infected with the second larval stage (the tetrathyridium) of the cestode M. corti by an intraperitoneal injection. Tetrathyridia invade the liver within 4 days of infection. They stimulate T-cells which trigger an inflammatory reaction with collagen deposition after the 10th day post infection. This reaction is absent in mice genetically lacking a thymus (athymic mice) but can be restored by a thymus or spleen cell graft.

Tetrathyridia multiply at a normal rate during the first 10 days of infection in mice in which the immune system has been inactivated by gamma irradiation. Cell grafts from normal mice to irradiated mice effect tetrathyridial multiplication. Cells derived from the thymus, or peritoneal exudate T-cells tend to stimulate tetrathyridial multiplication, while cells from lymph nodes, spleen, peritoneal cavity, bone marrow and peritoneal B-cells tend to inhibit tetrathyridial multiplication.

A study on the cellular changes occurring in the peritoneal cavity following an intraperitoneal injection of tetrathyridia shows that the lymphocyte population decreases while the numbers of macrophages, eosinophils and neutrophils increases.

The mouse produces antibodies against the parasite which can be detected by various tests. Positive results are obtained against the somatic antigen, using the Ouchterlony gel diffusion test and the indirect haemagglutination test, from week 3 and 9 post infection respectively. The passive cutaneous anaphylaxis test shows the presence of IgE antibodies against both antigens on the 12th week post infection. The indirect fluorescence antibody technique shows the presence of antibodies which bind to the interior structures of the tetrathyridia from either normal or athymic mice. This test also reveals the presence of host protein laid down around the parasite present in the peritoneal cavity of normal mice. Mouse antibody forming cells secreting antibodies to the

tetrathyridial excretory and secretory antigen can be demonstrated by the plaque forming cell technique. The antibody detected during the first 4 weeks of infection is of the IgM type. Plaque forming cells are most numerous in the spleen of infected mice.

The transfer of serum from donor mice, which have been infected for 12 weeks, to recipients at the time of infection by an intraperitoneal injection, lowers tetrathyridial multiplication in both normal and athymic recipient mice.

Partial protection against tetrathyridial infections is transferred to mice by a primary infection with irradiated parasites (80 k Rads). However mice vaccinated with the bacillus Bacille Calmette-Guerin show no protection against M. corti tetrathyridia.

INTRODUCTION

Mesocestoides corti is a Cyclophyllidean cestode which becomes sexually mature in the intestines of carnivores (Eckert, Brand and Voge, 1969). The first larval stage developes from eggs passed with the host's faeces in oribatid mites. The second larval stage, referred to as a tetrathyridium, developes in the viscera of both reptiles and mammals. This thesis presents a study of the immunological responses of the mouse to proliferating tetrathyridia.

Tetrathyridia have a simple granular body with a scolex bearing 4 suckers. They multiply asexually, either by producing lateral outgrowths of the body, posterior to the scolex, or by fission of the body starting at the scolex (Specht and Voge, 1965).

All the experimental mice used were infected by an intraperitoneal injection of a suspension of tetrathyridia in Kreb's Ringer saline. A stock of tetrathyridia was maintained by serial passage in mice. Tetrathyridia infect the liver within 4 days of entering the peritoneal cavity, in which they migrate at random and start to multiply (Specht and Widmer, 1972). Some of the parasites escape from the liver returning to the peritoneal cavity, where they continue to proliferate (Specht and Voge, 1965). After the fifth week of infection, some of the liver tetrathyridia penetrate the diaphragm and infect the lungs (Pollacco, 1975).

The first reported immunological study of this parasite in mice was published by Kowalski and Thorson (1972a). They demonstrated that serum taken from mice, which had been infected for 3 months, could confer a measure of passive immunity to other mice reducing the rate of multiplication of the parasite (1972a). They also found they could give a similar partial immunity to mice by repeated prior subcutaneous injections with the excretory and secretory products of the parasite (Kowalski and Thorson, 1972b). Their work was expanded by Kazacos (1976), who showed that mice could be immunized by subcutaneous implantation of the parasite. I have successfully repeated Kowalski and Thorson and Kazacos' experiments myself

(Pollacco, 1975). However, inspite of an immunological reaction in the mouse to the parasite, the mouse eventually succumbs to the heavy parasite burden, after about 8 months to 1 year.

Specht and Widmer (1972) have shown, in a detailed study of the pathology of the mouse liver infected with M.corti, that the migration of the tetrathyridia is initially limited by collagen formation and an extensive inflammatory reaction. Total encapsulation of the parasite occurs after the 8th week of infection and they suggest that this may be due to an antigen-antibody reaction involving penetration enzymes.

Preliminary experiments (Pollacco, 1975) showed that by comparison, the multiplication of the parasite in mice genetically lacking a thymus is much faster than in normal mice, up to 20 days post infection (athymic mice do not live more than 3 to 4 weeks under normal laboratory conditions). Also it was found that tetrathyridial multiplication is decreased in infected normal mice receiving spleen cell grafts from 3 months infected donor mice of same inbred strain (Pollacco, 1975). Thus M. corti may resemble several other helminths in which cell-mediated immunity (CMI) has been shown to play a critical role in the host parasite relationships. However the transfer of resistance by cellular transplantation could be due to a major contribution of antibody forming cells. This suggests that the inhibition of tetrathyridial multiplication, is in some way dependent on cellular immunity.

Evidence that lymphoid cells may play a protective role in immunity against certain parasites was reported by Larsh, Goulson and Weatherly in 1964 (a & b). They demonstrated adoptive immunity in mice to Trichinella spiralis following the transfer of lymph node cells and peritoneal exudate cells from mice infected with this nematode. More information about CMI has come from studies on the nematodes Trichostrongylus colubriformis and Nippostrongylus brasiliensis (Larsh and Weatherly, 1975). Some work on cellular immunity was also carried out with trematodes. Colley, in 1971, showed that lymph node cells from mice infected with Schistosoma

mansoni were transformed to blast cells in vitro, when exposed to a soluble egg antigen. This work was further expanded by Colley himself and other workers (Larsh and Weatherly, 1975).

The cestode on which most cellular work has been done is Hymenolepis nana. Thymectomised newborn mice harboured more cysticercoids from a challenging infection that intact sensitised controls. However grafting pieces of thymus tissue back into the same thymectomised mice restored the immunological response to normal (Okamoto, 1968). The use of immunologically suppressive chemicals (e.g. cortisone) has shown suppression of immunity in both Hymenolepis microstoma (Ritterson, 1971) and Hymenolepis diminuta (Hopkins, Subramanian and Stallard, 1972).

These experiments provide evidence for the critical role of CMI in some parasitic infections. However, there are other helminths which lend themselves to experiments on the mechanism of CMI. M. corti infections in mice have given some evidence that cellular immunity plays a critical role in restricting parasite proliferation, and offers a good experimental system with which to further study the host's cellular reaction to the parasite.

Kazacos' (1976) work, indicates that immunity mainly acts in the liver, because the significant difference in tethrathyridial multiplication between normal and immunized mice occurs at this site. Also, as described in this work, the numbers of recovered tetrathyridia differ between normal and athymic mice due to the increased multiplication rate of the parasite in the liver. Thus a histological comparison of the liver pathology in infected normal mice, athymic mice, and athymic mice reconstituted with thymus-cell grafts, may point to the causes of the difference in the rate of parasite multiplication, and, by inference, which responses are T-cell dependent. The effects of cellular grafts from different organs when transplanted into mice irradiated with gamma rays so as to inactivate the whole immune system, or into athymic mice, offers a means of associating effective inhibition of tetrathyridial multiplication with particular cell populations.

Kowalski and Thorson (1972a) reported that the immunization of mice with serum from 12 weeks infected mice gave a degree of protection to normal mice, but did not report the types of antibodies present in the serum. Serological tests identifying the classes of antibodies present are described and, from the association between the antibodies present and the degree of protection conferred by that serum to normal mice, the nature of protective antibodies can be inferred. An attempt to passively immunize athymic mice is also described in this thesis to show whether antibodies can act against the parasite in the absence of T cells.

A differential cell count of abdominal peritoneal fluid may be an aid to the study of the total body response to ageing, hormones and disease (McGowan and Davis, 1969). Since tetrathyridia infect the peritoneal cavity of mice a study is described to determine the cellular changes occurring, as a result of the infection, in the mouse peritoneal fluid during the first 12 weeks of the infection period.

Attempts carried out by Kowalski and Thorson (1972b) and by Kazacos (1976) to immunize mice, only transferred a partial immunity to recipient infected mice. In this thesis various attempts are described to induce total resistance in the mouse against a challenging infection.

a) Tetrathyridial multiplication is inhibited by exposing the parasite to γ-irradiation from a ^{60}Co-source (Pollacco, 1975). An experiment is described, designed to study whether the mouse infected with such tetrathyridia can develop a protective immunity effectively resisting a second infection.

b) It has been shown that the injection of Bacille Calmette-Guerin (BCG) suppresses tumour growth in both experimental animals and man. It has not yet been clearly understood how BCG confers protection but it has been postulated that increased macrophage activity is responsible for the greater resistance. Wolfe, Tracey and Henney (1976) speculate that BCG acts via a non-macrophage population of cytotoxic cells. Clark, Allison and Cox (1976) protected

mice against Babesia microti and Babesia rodhani by vaccination with BCG. They suggest that BCG acts by increasing the release of non antibody, soluble mediators of immunity. An attempted study is reported to test whether mice can be immunized with BCG against tetrathyridial infections.

The experiments described in this thesis investigate the following aspects of the mouse's immune response to M. corti:-

1. The role of the T-lymphocytes on tetrathyridial multiplication in mice. Genetically athymic mice, normal mice of the same inbred strain, and athymic mice reconstituted by cell transplants have been used in the experiments described.
2. The effect of cell transplants in intact, irradiated and genetically athymic mice on the multiplication of the parasite.
3. Changes in the cell population of the peritoneal cavity of infected mice and their significance on the host's response.
4. The classes of antibodies detectable in the serum and types of antibody forming cells in various tissues of infected mice.
5. The effects of passive immunization with serum of normal and athymic mice against the parasite.
6. The effects of immunizing mice against tetrathyridial infections by infecting the mice with irradiated parasite; or by treating with BCG.

MATERIALS AND METHODS

Animals

a) MICE: Mice from the following strains were used:

i) Quackenbusch and H mice, both outbred strains, were used because they were routinely bred in the Zoology Department.

ii) CBA/H mice, an inbred strain, were used for cell transfer experiments, and were supplied by the John Curtin School of Medical Research, Australian National University (JCSMR, ANU).

iii) Female athymic BALB/c mice were used in some of the experiments reported in this thesis. These mice fail to develop a thymus and are termed 'nude' mice because they also lack hair. Athymic mice are homozygous for a lethal gene (i.e. nu/nu) and are obtained by crossing nu/+ mice. Female athymic T-cell reconstituted, obtained by injecting a suspension of thymus cells from 5 normal BALB/c mice intraperitoneally into athymic mice, were also used. Female intact BALB/c mice (nu/+) were used as experimental controls.

All these BALB/c mice were supplied by Dr Graham Mitchell of the Walter and Eliza Hall Institute, Melbourne. They were bred and reared up to 6 to 8 weeks of age at the Institute in a specific pathogen-free laboratory. In my laboratory, they survived for 3 to 4 weeks if kept away from other mice and in a warm place. All mice were maintained in my laboratory under conventional conditions and fed ad lib on Mecon rat and mouse cubes (Fidelity Feeds).

b) RATS: Wistar rats, supplied by JCSMR, ANU, age 12 to 15 weeks, were used.

The Parasite: Mesocestoides corti

A stock of parasitised mice of each of the strains in use was maintained to supply tetrathyridia for the experiments. These were infected by an intraperitoneal injection (needle:

22G 1¼") with 0.05 ml (approximately 250 larvae) of packed tetrathyridia. The infected mice survived for about 12 months before succumbing to the infection. The donor mice were killed by heavy anaesthesia, and the tetrathyridia were obtained by washing out the peritoneal cavity into petri dishes containing saline (Ringer's solution prepared from tablets: BDH). The tetrathyridia were washed 5 times with saline prior to injection into recipient mice. For experiments, mice were infected by intraperitoneal injection of freshly harvested tetrathyridia from donor mice of the same strain as the recipients.

Parasite Recovery:

The numbers of tetrathyridia present in experimental mice were determined by killing the mice and counting separately those present in the peritoneal cavity and the liver. The tetrathyridia in the peritoneal cavity were washed into petri dishes and counted. To recover tetrathyridia from the liver, the organ had to be finely minced and then digested with trypsin. Each finely chopped liver was digested in 13 ml of 1% trypsin (CSL, Melbourne, Australia) at 37° for 5 to 6 hours, with continuous shaking. The tetrathyridia were counted in petri dishes under a dissecting microscope.

Preparation of antigens of M. corti

Two types of antigens were prepared.

a) SOMATIC ANTIGEN (technique after Kowalski and Thorson, 1972b)

Tetrathyrdia from the peritoneal cavity of heavily infected mice were obtained as described above. These were placed in a glass homogeniser with an equal volume of 0.85% NaCl and homogenised, using an electric driven motor, until suspension looked homogenous. The homogeniser tube was cooled on ice at frequent intervals to reduce protein denaturation. The suspension was centrifuged at 3900 g in a cold room for 30 minutes. The supernatant was centrifuged again for 30 minutes, the pellet discarded, and protein in solution was estimated using the Lowry (1951) method. The supernatant was aliquoted in small amounts according to anticipated use and stored at -20°C.

b) EXCRETORY AND SECRETORY ANTIGEN (technique after Kowalski and Thorson, 1972b)

Tetrathyridia were obtained from the peritoneal cavity of infected mice, as described above, using aseptic measures (i.e. using sterile instruments, glassware and saline). After washing with sterile saline, 1 ml of packed tetrathyridia was placed in a 150 ml sterile screw capped bottle and 35 ml of sterile Kreb's Ringer phosphate solution (described below) added. These were incubated at 37°C for 3 days before replacing the medium. The medium was collected again after a further 2 days and the tetrathyridia were then discarded. Protein in the culture media was estimated by Lowry's method (1951) and concentrated by dialysis using Minicon B15 apparatus (Amicon Corporation) until the protein concentration was within the range required for various experiments. The concentrated solution referred to as E/S antigen was aliquoted and stored at -20°C until used. For both antigens, the protein concentration was taken as a measure of the antigen concentration.

Protein Estimation: after Lowry, Rosebrough, Farr and Randall (1951)

Reagent A 2% Na_2CO_4 in 0.1 M NaOH.
Reagent B 10% $CuSO_4.5H_2O$ and 2% Na or K tartrate.
Reagent C 50 ml A added to 1 ml B (prepared before use).
Reagent D Folin reagent (BDH) diluted 1:2 before use.

A standard curve of known protein concentration was prepared from bovine serum albumen (BSA) with each protein determination. BSA solution (1 mg/ml) was diluted to give final protein concentration of 50, 100, 150 and 200 µg/ml. All tubes were brought to 1 ml with distilled water and a blank containing 1 ml distilled water was also prepared. To each tube 4 ml of Reagent C and after 10 minutes 0.4 ml of Reagent D were added. Thirty minutes later the optical density of the solutions was measured spectrophotometrically (Unicam SP600 Series 2) at 750 nm. The protein concentration of the prepared antigens was calculated from a graph of absorbance against the known concentrations.

Kreb's Ringer Phosphate Solution:

This culture medium was prepared as described by Long (1961).

Compounds	g	Distilled water (mls)
NaCl	8.460	940
KCl	0.460	40
$CaCl_2$	0.183	30
KH_2PO_4	0.211	10
$MgSO_4$	0.382	10
Na_2HPO_4	4.607	130
$NaHCO_3$	1.820	140
Glucose	1.3	
Phenol red	0.026	

All the solutions except the $CaCl_2$ and the $MgSO_4$ were mixed together with the glucose and phenol red. The three final solutions were autoclaved separately and then mixed. The solution was bubbled with a gas mixture of 5% CO_2 in air until brought to pH 7.2 as indicated by the phenol red indicator (becomes cherry red in colour).

Serum Collection:

The mice were deeply anaesthetised and an incision was made in the skin across the ventral side of the thorax. The skin was folded back exposing the axilla on one side and the subclavian artery and vein were severed. The blood which collected in the axilla was quickly transferred by a pasteur pipette to a glass centrifuge tube. The blood was allowed to clot at room temperature for about 60 minutes before freeing the clot, with a wooden stick, from the tube. The blood was kept at 4°C overnight and then centrifuged at 980 g for 20 minutes. The supernatant was recentrifuged for 30 minutes and the serum aliquoted in small amounts and stored at -20°C until used.

Serological Tests:

Some serological tests were carried out to detect antibodies in the serum of mice parasitised with tetrathyridia of M. corti. The sera were tested by four different techniques

to identify different types of antibodies present against the two prepared antigens: (i) somatic antigen, and (ii) the excretory and secretory antigen (ES). The tests carried out were the following:

(A) Indirect Haemagglutination (technique after Ali-Khan, 1974)

Agglutinating antibodies to tetrathyridial antigens (somatic or ES) present in mouse serum were identified by this test. The specific antigen was coupled on to sheep red blood cells (SRBC), using glutaraldehyde as a fixing reagent. These cells were incubated with serum at room temperature for several hours in microtitre plates and the results were read according to Statvisky and Arquilla (1955). Agglutinating antibodies, which are mainly multivalent, react (by their Fab moiety) with the antigen which has been attached to the SRBC, causing the red cells to spread out in a loose meshwork and form a carpet of red cells. In the absence of agglutinating antibodies no reaction occurs and the SRBC settle in a closely packed button of red cells.

Coupling antigen to sheep red blood cells with glutaraldehyde for Indirect Haemagglutination:

Sheep red blood cells (SRBC) in Alsever's solution were supplied by the John Curtin School of Medical Research (JCSMR). The cells were washed five times with physiological saline by centrifugation at 980 g in a bench centrifuge. Glutaraldehyde was used to bind the antigens, either, ES or somatic antigen (prepared as described above) to the SRBC. The process was carried out in the following steps:

(i) A suspension of 3% (v/v) of SRBC in 0.85% NaCl was prepared and 1.5 ml of this was mixed with 1 ml of either antigen at a concentration of 3 mg protein/ml. To this mixture, 1.5 ml of 0.029% glutaraldehyde solution (Ajax Chemicals Ltd, Australia) in 0.85% NaCl, buffered with 0.1 M phosphate pH 8.2 was added drop by drop while agitating the mixture.

(ii) The mixture was left for one hour at $4^{\circ}C$, then the SRBC were washed three times with 0.85% NaCl and 4 times

with distilled water by centrifugation.

(iii) The SRBC were resuspended after the final wash in 4 ml of 0.2% aqueous merthiolate (preservative).

(iv) For Controls, antigen-free SRBC were prepared by repeating the above 3 steps but replacing the addition of antigen in step (i) with 0.85% NaCl.

Antigen-coated SRBC were kept for up to six months at $4^{o}C$ before use, but were washed once in 0.85% NaCl before use as a 1% (v/v) suspension. The cells were used in tests as a 1% (v/v) suspension in 0.85% NaCl.

Preparation of sera for indirect haemagglutination:

All sera to be tested were absorbed for 10 minutes at room temperature with an equal volume of packed SRBC (previously washed as above) to absorb any natural antibody present to sheep antigen. After absorption, the SRBC were spun down at 980 g for 10 minutes, and the pellet of red cells formed discarded: sera were ready to be tested. The actual test was carried out in microtitre plates as described in Diagram 1. A serial dilution of serum in 0.85% NaCl (diluent) was carried out from well 2 to well 10. After the addition of all the solutions, the plates were sealed with parafilm and gently mixed by light agitation, and left at room temperature for several hours. The results were then read and scored.

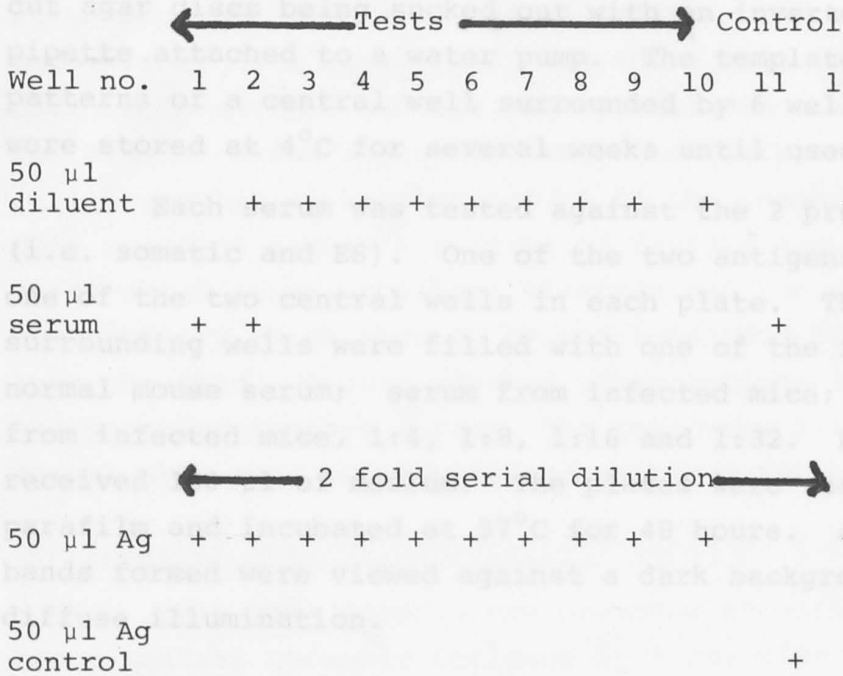
(B) Ouchterlony Gel Diffusion Test: (modified technique as used by Campbell, Garvey, Cremer and Sussdorf, 1970)

This test detects the presence of precipitating antibodies in the serum a particular antigen. It was carried out in agar plates in which a pattern of wells were punched to hold the tested media. Both sera and antigens diffuse through the agar in opposite directions and where the precipitating antibodies and the antigen are both present at equivalent concentrations a precipitation band developes in the agar.

Preparation of gel plates for double diffusion technique:

The following mixture was autoclaved and poured into 9 cm diameter disposable sterile petri dishes while still molten.

Diagram 1 - Indirect Haemagglutination; arrangement of test solutions



Ag test: Somatic or ES antigen coated SRBC

Ag control: Control SRBC without antigen

1 g Agar-agar (Ajax Limited)
100 ml veronal buffer (LKB) veronal buffer solution)
0.01 g sodium azide

When the agar had solidified at room temperature a pattern of holes (wells) was punched using a template, the cut agar discs being sucked out with an inverted pasteur pipette attached to a water pump. The template gave two patterns of a central well surrounded by 6 wells. The plates were stored at 4°C for several weeks until used.

Each serum was tested against the 2 prepared antigens (i.e. somatic and ES). One of the two antigens was placed in one of the two central wells in each plate. The six surrounding wells were filled with one of the following: normal mouse serum; serum from infected mice; diluted serum from infected mice, 1:4, 1:8, 1:16 and 1:32. Each well received 100 µl of medium. The plates were sealed with parafilm and incubated at 37°C for 48 hours. Any precipitating bands formed were viewed against a dark background using diffuse illumination.

(C) Passive Cutaneous Anaphylaxis: (technique after Weir, 1973)

Passive cutaneous anaphylaxis (PCA) tests for IgE antibodies. It is carried out on live animals of a closely related species to the animal from which the serum to be tested has been taken.

Mouse serum was tested by the PCA reaction in rats. Sera from infected mice and control sera from non-infected mice were tested. A further control serum was made by heating serum from infected mice for 30 minutes at 56°C in a water bath, since IgE is relatively heat sensitive. Six Wistar male rats of about 15 weeks of age were used. Their backs were shaved and 5 places marked with a green or red felt pen. An intradermal injection was made into each position, using a 26 G ½" needle, of 0.05 ml of one of the following: undiluted serum, serum diluted 1:3 and 1:9 with saline, or one of the 2 control sera (as above). The site of injection was encircled with a felt pen. Any IgE antibodies present in the tested sera will attach to mast cells present at the site of injection.

Twenty-four hours later, the rats were injected intravenously (using 26 G ½" needle) via the tail vein with a mixture of 0.2 ml of either ES or somatic antigen (at a protein concentration of 6.25% (w/v) in saline) plus 0.5 ml of Evans blue (at a concentration of 24% (w/v) in saline). The rat tails were scrubbed clean and to facilitate the injection, the rats were warmed under an infra red lamp, then placed in a restrainer cage while injection was carried out. Both antigen and dye are carried via the circulation to the site of serum injection, where in the presence of mast cell boung IgE a reaction occurs to the respective antigen. This reaction causes a release of factors from these mast cells which cause capillary dilation and increased capillary permeability, which shows up by the localised diffusion of dye into the skin. Twenty minutes after antigen injection, when the reaction reaches a peak, the rats were killed, their dorsal skin cut, reflected, and examined for blue areas.

(D) Indirect Immunofluorescence Technique: (after Weir, 1973)

This test detects the presence of antibodies in the serum against parasite antigens by a reaction made visible by fluorescence in UV light under the microscope. Both frozen and paraffin sections of parasite tissue were examined as well as paraffin sections of infected liver tissue. These sections were incubated with serum from normal or parasitised mice, to allow mouse antibody if present to bind to the parasite. Any mouse antibody attaching on to the tissue examined was revealed, after washing away the mouse serum, by addition of rabbit antimouse immunoglobulin conjugated with fluorescein isothiocyanate (Nutritional Biochemical Corp. USA). When the sections were viewed under the ultra violet (UV) microscope (Leitz) with appropriate excitatory and barrier filters the presence of fluorescent antibody is clearly visible.

Preparation of Sections:

a) Frozen sections of peritoneal tetrathyridia were prepared from 3 week infected normal and athymic female BALB/c mice. Tetrathyridia were immersed in Tissue-Tek (Lab-Tek Products,

Illinois) diluted 1:2 with water, then frozen in a stream of carbon dioxide in a freezing microtome. Sections 7 µm were cut, dried in air, fixed in 100% ethanol for 30 minutes, and then dried at room temperature. They were stored at 4°C till used.

b) Paraffin sections of peritoneal tetrathyridia and infected liver tissue from 3 week infected normal and athymic female Balb/C mice were prepared as follows:

i) Tissue was fixed in 95% ethanol at 4°C for 24 hours.

ii) It was then transferred to cold absolute ethanol, with 3 changes in 1 hour.

iii) The tissue was placed in cold xylol, with 2 changes in 1 hour, and then left at room temperature for 1 hour, or stored at 4°C for 1 to 2 days before embedding.

iv) The tissue was blocked in paraffin wax and stored at 4°C till sectioned.

v) Sections of 7 µm were cut on a microtome and picked up on glass slides.

vi) Before carrying out fluorescence antibody test, the slides were dewaxed by passing them through 2 changes of absolute ethanol, and one change of 80%, 70% and 50% ethanol.

The sections, prepared by either freezing or the wax method were passed through 3 changes of 5 to 10 minutes each, of Dulbecco phosphate buffered saline (explained below). The area around the sections was dried with a tissue and the sections covered with several drops of normal or 'immune' serum (the latter obtained from 12 week infected CBA male mice) or fluorescent immunoglobulin solution (rabbit antimouse immunoglobulin, diluted 1:16 with 0.02% Evans blue in PBS) as explained in Table 1. The sections were incubated on a damp filter paper under a petri dish for 20 minutes and then washed 3 times in Dulbecco's buffer as above. The sections incubated in the serum, after the buffer washings, were reincubated with the fluorescent immunoglobulin and then rewashed as above. All sections were mounted in 50% glycerol in water under a coverslip and stored

TABLE 1 - Tests carried out to detect the presence of antibodies in mouse serum to M. corti tetrathyridia antigens by indirect fluorescent antibody technique. Tetrathyridia were obtained from normal mice infected for 3 weeks.

Tissue	Preparation of sections	Serum	Fluorescence immunoglobulin
Peritoneal tetrathyridia	Frozen	-	-
Peritoneal tetrathyridia	Frozen	-	+
Peritoneal tetrathyridia	Frozen	Normal	+
Peritoneal tetrathyridia	Frozen	Immune	+
Peritoneal tetrathyridia	Wax	-	-
Peritoneal tetrathyridia	Wax	-	+
Peritoneal tetrathyridia	Wax	Normal	+
Peritoneal tetrathyridia	Wax	Immune	+
Infected liver tissue	Wax	-	-
Infected liver tissue	Wax	-	+
Infected liver tissue	Wax	Normal	+
Infected Liver tissue	Wax	Immune	+

These tests were repeated using peritoneal tetrathyridia and liver tissue from 3 week infected athymic mice.

- means none added; + means added

Normal serum obtained from uninfected CBA/H male mice.

Immune serum obtained from 12 week infected CBA/H male mice.

in a closed plastic box over wet paper. The mounted preparations were viewed under a Leitz Wetzlar ultra violet fluorescence microscope. A 200 W mercury vapour lamp and B G 12 excitation filters plus UV absolute barrier filter were fitted in the microscope. For photography a Kodak photomicrography monichrome (SO410) for black and white and a high speed ektachrome daylight film for colour were used.

Preparation of Dulbecco's phosphate buffered saline:

The following 3 solutions are prepared:

A.		B.		C.	
Compound	Weight (g)	Compound	Weight (g)	Compound	Weight (g)
NaCl	8	$CaCl_2$	0.1	$MgCl_2 6H_2O$	0.1
KCl	0.2	Glass distilled water to 100 ml		Glass distilled water to 100 ml	
Na_2HPO_4	1.15				
KH_2PO_4	0.2				
Glass distilled water to 800 ml					

Each solution is prepared separately and then mixed.

Cellular Transplants:

An attempt was made to study the role of different populations of cells against the tetrathyridial stage of M. corti in mice. The cellular suspension were obtained from inbred normal, BALB/c or CBA/H mice and transplanted into athymic (BALB/c) or irradiated (CBA/H) mice (described below) of the same respective inbred strain. Cell suspensions from spleen, thymus, bone marrow, lymph node and peritoneal cavity were prepared as follows (after Weir, 1973):

a) Spleen Cells

Mice were killed and their spleens removed. The spleen was forced through a phosphobronze wire mesh (250 µ) into a petri dish containing Hanks balanced salt solution (BSS)

(CSL, Melbourne), using a plastic syringe plunge. Cell clumps were broken up by mixing the cell suspension with a pasteur pipette. The suspension was centrifuged in glass centrifuge tubes at 350 g for 18 seconds to sediment cell clumps and debris, which were discarded. The supernatant was centrifuged at 350 g for 7 minutes. The cell pellet was resuspended in a suitable volume of Hanks BSS, and the number of visible nucleated cells counted in a haemocytometer, using 1:10 dilutions of the cell suspension in white cell diluting fluid (0.01% trypan blue in 5% acetic acid). Cells were kept on ice till used.

b) Thymus cells

The thorax was cut open ventrally by making an incision across the lower border of the rib cage. The heart and thymus were then exposed by making 2 lateral incisions and lifting up the cut ribcage with forceps. The thymus was then freed from the thoracic wall with blunt ended scissors, and the lower 2/3 of the thymus, overlying the heart were cut off. The upper 1/3 of the organ was not used to avoid the parathymic lymph nodes which are situated very close to the thymus. The thymus was forced through a phosphobronze wire mesh (as for spleen). Cell clumps were broken up with a pasteur pipette and the cells centrifuged down at 350 g for 7 minutes. The pellet was resuspended in alkaline Hanks BSS (made by adding 1 drop of IN-NaOH to 100 ml Hanks BSS). In an alkaline solution dead cells and cellular debris clump, so that these may be eliminated by centrifugation at 350 g for 10 seconds. This minimizes loss of lymphoid cells. The supernatant was recentrifuged at 350 g for 7 minutes and the cell pellet resuspended in an appropriate volume of Hanks BSS solution. The numbers of visible lymphoid cells were counted as described above for spleen cells.

c) Bone marrow cells

Mice were killed and their hind legs removed. The femur was freed from skin and muscle, and both ends of the bone were cut with a scalpel. Hanks BSS was forced through

each end of the femur using a syringe fitted with a 26G ½" needle. The bone marrow cells, which are extruded from the other end of the bone were collected in a petri dish containing Hanks BSS solution. The bone marrow cells were dispersed with a pasteur pipette and suspension of lymphoid cells was prepared and counted as described for the thymus cells.

d) Lymph node cells

The mice were killed and cut open by making a mid-ventral incision. The sternal, mesenteric and hepatic lymph nodes were removed and a suspension of lymphoid cells prepared and counted as described for spleen cells.

e) Peritoneal cells

Mice were killed by cervical dislocation to minimize haemorrhage into peritoneal cavity. The skin overlaying the peritoneal cavity was removed gently to prevent any damage to the underlying body wall. 8 ml of Hanks BSS were injected intraperitoneally, using two 5 ml syringes fitted with 22G 1¼" needles, each syringe inserted on either side of the mouse. The intestines were gently palpated so as to disperse the cells and the fluid was then withdrawn into the syringes. The cell suspension was centrifuged at 350 g for 7 minutes, the supernatant discarded and pellet resuspended in an appropriate volume of Hanks BSS. The numbers of cells were counted (as for spleen cells). With infected mice, any tetrathyridia sucked up with the peritoneal cell suspension into the syringe, were removed before centrifugation.

Fractionation of Peritoneal Cell Suspensions

In some experiments the differential effects on tetrathyridial multiplication by the varied cell populations present in a peritoneal cell suspension were studied. Peritoneal cell suspensions obtained as explained above were separated into 3 different populations:

a) Non-phagocytic peritoneal cells
b) Peritoneal B-cells
c) Peritoneal T-cells

These cell populations were transplanted separately, or combined, into recipient mice infected at the same time as cell transfer.

a) Non-phagocytic peritoneal cells

A method developed by Rous and Beard (1934) and adapted by Parish and Hayward (1974a) was used to deplete the number of phagocytic cells in peritoneal suspension. I am grateful to Dr C. Parish (JCSMR, ANU) who demonstrated the method.

1. Peritoneal cells were obtained as explained above and a cell suspension containing 10-20 x 10^6 cells/ml in Hanks BSS - 10% Fetal calf serum (FCS) (CSL, Melbourne) was incubated with sufficient carbonyl iron powder (supplied by JCSMR) to render the solution a dark grey colour. The suspension was slowly rotated for 30 minutes at 37°C to facilitate phagocytosis.
2. The depletion of phagocytes occurs by either the cells taking up the iron filings (phagocytosis) or by an iron filing adhering to them (adherence). Any remaining free iron particles were removed from the suspension by slowly passing the tube 4 times between the poles of a strong electro magnet.
3. The supernatant containing the peritoneal cell suspension, free of most of the phagocytic cells, was retained, and the pellet formed by magnetisation was discarded.

b) Separation of T-lymphocytes and B-lymphocytes

Peritoneal non-phagocytic cells are mainly lymphocytes of both T- and B-type. These were separated by using a "rosetting" procedure, as described by Parish and Hayward (1974a). The technique was carried out with the assistance of Dr Parish (JCSMR).

1. Ten µl of rabbit anti-mouse immunoglobulin (kindly donated by Dr Parish, JCSMR), was added to 2.7 ml of lymphocytes (2 x 10^7 cells/ml before removal of phagocytic cells) in Hanks BSS - 10% FCS. The mixture was shaken (using an electrical agitator) and left on ice for 30 minutes. Since

only B-cells have mouse immunoglobulin (Ig) on their surface, the rabbit anti-mouse Ig will only adhere to the B-cells. The suspension was centrifuged at 350 g to bring down both the T- and B-cells, which were re-suspended in 1.25 ml of Hanks BSS - 10% FCS.

2. Chromic chloride was used to chemically attach anti-rabbit Ig to sheep red blood cells. Ten µl of sheep anti-rabbit Ig (kindly donated by Dr. Parish, JCSMR) were mixed with 4 ml 0.85% NaCl and 0.25 ml packed sheep red blood cells (SRBC). To this was added 0.22 ml of 0.1% $CrCl_3$ (adjusted to pH 5) while the mixture was continuously shaken. The mixture was allowed to stand for 10 minutes at room temperature and then 5 ml of phosphate buffered saline were added to stop the reaction. The cells were centrifuged, washed once in phosphate buffered saline, (PBS) pH7, and resuspended in 1 ml PBS. The cells were kept on ice till used.

3. The lymphocyte suspension, containing B-cells, coated with rabbit anti-mouse Ig, were mixed with the SRBC coated with sheep anti-rabbit Ig, and then centrifuged for 5 minutes at 630 g in a cold room. The sheep anti-rabbit Ig on the SRBC reacts with the rabbit anti-mouse Ig on the B-lymphocyte, forming "rosettes" of red cells around each B-cell. T-lymphocytes, which do not bind anti-mouse Ig (lacking appropriate surface Ig molecules), do not form "rosettes". Ten µl of 25% sodium azide was then added to prevent formed rosettes from falling apart and improve cell separation at 20°C. The cell pellet was very gently resuspended and the suspension was then left to warm up to room temperature.

4. The cell population was fractionated by layering the suspension on a solution of a high molecular weight, contained in tubes (15 mm diameter, with U-shaped bottoms), followed by centrifugation. As a separating medium Isopaque (a derivative of iodobenzoic acid)/Ficoll (a polyglucose) was used. The SRBC lymphocyte mixture was gently layered on top of the Isopaque/Ficoll column. The tube was placed

in a centrifuge, prewarmed to 20°C, and centrifuged at 1200 g for 30 minutes. The centrifuge used is one that accelerates rapidly and attained 1200 g within a few seconds. Before centrifuging, a small sample was taken for counting developing "rosettes". (explained in step 7).

5. After centrifugation, the supernatant above the Isopaque/Ficoll interface was discarded and the lymphocyte layer, together with all the separating media above the red cell pellet (containing the T-cells) was collected. The lymphocyte preparation ('upper layers') was centrifuged at 630 g for 5 minutes and resuspended in 10 ml Hanks BSS - 10% FCS. The cells were washed twice and then resuspended in 0.5 ml Hanks BSS - 10% FCS and counted in a haemocytometer as previously described.
6. A solution which lyses red cells was added to the red cell pellet (lower layers, containing the B-cells). This was prepared from a stock solution before use:

Solution A	Weight (g)
NaCl	15.56
KCl	1.48
$CaCl_2$ (anhydrous)	0.67
$MgCl_2 6H_2O$	1.22
Double distilled water	100 ml

Solution B	Weight (g)
i) $NaH_2PO_4 2H_2O$	0.312
Double distilled water	100 ml
ii) $Na_2HPO_4 2H_2O$	0.356
Double distilled water	100 ml
Mix 90 ml of i) with 10 ml of ii)	

Solution C	
Solution A	100 ml
Solution B	100 ml
25% NaN_3 (sodium azide)	0.04 ml

Store in fridge. Filter if necessary.

Solution D	
Solution C	0.5 ml
Double distilled water	11.25 ml

To the red cell pellet, 8.55 ml of solution D was added and pellet rapidly resuspended, using a 10 ml pipette. To restore isotonicity, 1 ml of solution C was added, mixed and the suspension centrifuged at 630 g for 5 minutes. The cells were then resuspended in 1 ml Hanks BSS - 10% FCS for counting in a haemocytometer slide.

7. The number of "rosettes" formed in the lymphocyte suspension was counted before fractionating the cell population as follows. To 10 µl of the "rosette" suspension, 0.2 ml of 2% methyl violet in normal saline and 0.19 ml Hanks BSS - 10% FCS were added. All the lymphocytes take up the dye. The T-cells can be recognised because they occur singly, while the B-cells occur with a cluster of red cells around them (i.e. rosette formation). Rosettes were counted on a haemocytometer slide and the percentage of rosette-forming cells to the total population of lymphocytes was calculated.

Phosphate buffered saline:

Phosphate buffered saline (PBS) was prepared from the following 2 solutions:

a) 0.1 M KH_2PO_4 i.e. 13.15 g in 1 litre distilled water
b) 0.1 M Na_2HPO_4 i.e. 14.19 g in 1 litre distilled water

For a buffer of pH 7, 39 ml of solution A were mixed with 61 ml of solution B.

Irradiation of mice and of M. corti tetrathyridia:

Both mice and tetrathyridia were irradiated with γ-rays from the ^{60}Co source at C.S.I.R.O. (Canberra). The γ-radiation declines daily through radioactive decay by a known factor. Thus, the required time of exposure to the ^{60}Co source was calculated each time to give the required dose of radiation. Also a correction factor was calculated to allow for the distance from the source to the centre of material being irradiated. The dose rate, in Rads per minute, was computed monthly by the officer at C.S.I.R.O. responsible for the facility, for specified distances from the source. After calculations had been made and the specimens placed in the

shielded radiation chamber, the ^{60}Co was raised from its shielded housing to the appropriate level for the calculated period of time.

i) Irradiation of mice:

Inbred CBA/H mice were irradiated with 750 Rads (a dose that inactivates most of the circulating lymphocytes (Weir, 1973) in groups of 5 to 7 in round tins (approximately 14 cm in diameter) containing small holes on their lids for the mice to breathe. These tins were placed on circular plates with 15 cm separating the source to the centre of the plates, (about 15 cm in diameter). These plates were rotated so as all the mice receive approximately the same dose of irradiation. Four hours after irradiation, the mice were infected and treated according to the particular experimental design.

ii) Irradiation of the parasite:

The parasite was irradiated with a dose of 80 k Rads (a dose that inhibits multiplication of tetrathyridia; Pollacco, 1975) in 3/4" diameter plastic tubes. Each tube contained 0.1 ml packed tetrathyridia in 3 ml Ringer's saline solution. The plastic tubes were placed in rotating metal tubes next to the source for the calculated period of time to give the above dose or irradiation. The irradiated tetrathyridia were pooled and immediately injected into the experimental mice.

Plaque-forming cell technique:

Plaque-forming cell technique (PFC) estimates the numbers of cells secreting antibodies to a specific antigen in a given organ. It can be adapted so as to distinguish cells secreting different classes of antibody. The method used has been described by Cunningham and Szenberg (1968) and is a modification of the Jerne (1963) PFC assay. The basis of the reaction is that, following an antibody-antigen reaction on the surface of a red blood cell, complement reacts with the complex and causes the red cell to lyse. The complement used was derived from guinea pig serum and the red cells were sheep red blood cells (SRBC). The antigen must be bound to the red cells prior to the reaction with the specific antibody.

Chromic chloride was used as the binding reagent. In the plaque assay, reactive lymphocytes are seen in microscope slide chambers in a field of closely packed red cells, surrounded by a clear space due to the lysis of nearby red cells.

Preparation of microscope slide chambers:

About 20 microscope slides (without ground glass ends) were afixed to a bench by running a ¼ inch 'Scotch' sticky tape on both sides, along the top and bottom ends of the row of slides arranged side by side. (The tape overlapped the edge of the slides so as to fix the slide to the bench). The slides were thoroughly cleaned with 100% ethanol and immediately wiped dry with a Kleenex tissue. Another length of tape was run down the centre of the row of slides. Another row of slides was laid down on top of the fixed, cleaned set of slides and pressed down very firmly. Each pair of slides provides 2 shallow chambers with a thickness equal to that of the tape. The pair of slides can be lifted off the bench, separated from each other, and stored in a clean box till used.

Fixing antigen to red cells:

Sheep red blood cells (SRBC) in Alsever's solution were supplied by the John Curtin School of Medical Research (JCSMR). The SRBC were washed five times in 0.85% NaCl by centrifugation at 980 g in a bench centrifuge. After the final wash, 0.25 ml of the packed cells were transferred to a large centrifuge tube (40 ml). Three ml of saline and 0.075 ml of antigen solution (either the excretory plus secretory antigen, or the somatic antigen) containing 3 mg protein per ml were added, or 0.075 ml of rabbit antimouse immunoglobulin (1:19 dilution; supplied by Dr Cunningham) was added. The choice depended on the reactant to be fixed on the SRBC. One ml of 0.01% chromic chloride (prepared as 0.1% solution, pH 5, and left to age for at least 3 weeks before use) was added drop by drop while shaking on a 'Vortex' agitator. The mixture was allowed to stand for 10 minutes at room temperature, and then

the SRBC were washed 3 times with 0.85% NaCl by centrifugation for 5 minutes at 980 g in a bench centrifuge. The SRBC were resuspended by agitation (Vortex) in 1 ml of saline and then mixed with a pasteur pipette with 11 ml 0.85% NaCl. After the last wash, the SRBC were left packed, some to be used to absorb complement, and the remainder diluted with 0.85% NaCl (containing streptomycin and penicillin) for the PFC assay. These antigen fixed cells can be stored in 0.85% NaCl (plus the antibiotics) at 4°C for up to 4 days before used.

Preparation of lymphocyte suspension:

Lymphocytes from spleen, bone marrow, thymus, lymph nodes and peritoneal cavity were obtained as explained previously. A lymphocyte suspension from lungs was prepared in a similar way to that from the spleen. For PFC assay, all lymphocyte suspensions were obtained from organs of Balb/C ♀ mice.

Absorption of anti-red cell antibody from Guinea pig serum:

It is necessary to remove any naturally occurring anti-SRBC antibody present in the guinea pig serum used to supply complement. One part of normal washed SRBC, or one part of the antigen-fixed SRBC (i.e. with ES or somatic antigen) were mixed with 3 parts of guinea pig serum (stored at -20°C) and allowed to stand for 10 minutes on ice. The cells were then centrifuged at 980 g for 5 minutes to remove the SRBC. The supernatant was used as the complement solution. This was distributed in small volumes and stored at -20°C.

Assay:

This PFC assay was initially demonstrated by Dr. Cunningham (JCSMR). For each assay, i.e. each different combination of specific lymphocyte suspension and specific antigen, the following were mixed in a well of a microtitre tray.

0.01 ml of complement solution
0.02 mls of antigen, or control SRBC without antigen, or rabbit antimouse immunoglobulin, fixed SRBC.

0.1 ml of lymphocyte suspension, or diluted 1:9 or 1:99 with Hanks BSS

0.01 ml of Hanks BSS, or developer

The developer is rabbit antimouse immunoglobulin (1:99 dilution; supplied by Dr Cunningham). The contents of each well were thoroughly mixed with a pasteur pipette and the suspension was pipetted into the 2 chambers of each microscope slide, filling them without bubbles. The gap between the slides was sealed by dipping the edges into molten paraffin wax. The slides were incubated for 55 minutes at 37°C and the number of plaques (appearing as clear zones) counted against a dark illuminated background.

Histology

(A) A study of the cellular and fibrogenic reactions occurring in livers infected with M. corti tetrathyridia

In the mouse, tetrathyridia multiply both in the liver and peritoneal cavity. However, the limitation of tetrathyridial multiplication (as indicated in experiment 2 - effect of cellular transplants on tetrathyridial multiplication) is shown to operate in the liver. A histological study was carried out on infected livers of both normal and athymic mice to investigate the role of the T-lymphocyte in controlling tetrathyridial multiplication.

a) Bouin's fixative

i) Fixed in Bouin's for 24 hours
ii) Washed in frequent changes of 70% ethanol for 2 days
iii) Dehydrated in 90% ethanol for 2 hours, then in 100% ethanol for 12 hours with several changes
iv) Cleared in xylene for 4 to 6 hours
v) Embedded in paraffin wax and blocked.

b) Carnoy's fixative

i) Fixed in Carnoy's fixative (60 ml 100% ethanol + 30 ml chloroform + 10 ml acetic acid) for 24 hours

ii) Washed in several changes of 100% ethanol for 6 to 24 hours
iii) Stored in 90% ethanol
iv) Brought to 100% ethanol for 8 to 24 hours with several changes
v) Cleared in xylene for 6 hours
vi) Embedded in paraffin wax and blocked.

The blocks were trimmed and 7 µm sections were cut and mounted on to glass slides. Different staining techniques were carried out to identify the host's cellular and fibrogenic response to the invading parasite.

a) Hematoxylin and Eosin (H and E)

Sections were stained with hematoxylin and counterstained with Eosin as described by Carleton (1957). They were dewaxed in xylene, passed through alcohol and washed in tap water. The sections were stained with Mayers acid hemalum for 30 minutes, rinsed in tap water and Scott's solution. The sections were then examined in tap water and restained and redifferentiated if necessary. After counterstaining in Eosin, sections were rinsed in tap water, passed through alcohol and xylene and then mounted in Canada Balsam.

b) Fast Green-van Gieson

Sections were dewaxed in xylene, passed through alcohol, washed in tap water, stained in hematoxylin and rinsed in tap water as above. The sections were then stained in 0.1% aqueous fast green for 4 minutes. The excess stain was washed in 1% acetic acid and the sections were then stained in 0.2% acid fuchsin in saturated aqueous picric acid for 10 to 15 minutes. The sections were rewashed in 1% acetic acid and then rinsed in distilled water, passed through alcohol and xylene, and mounted in Canada Balsam as above.

c) Picro-Ponceau with Hematoxylin

The staining procedure according to Humason (1972) was followed. The sections were dewaxed, passed through alcohol,

washed and stained in hematoxylin. They were then washed in running water until sections looked deep blue and then stained in picro-Ponceau. The tissue was rinsed, examined and restained and redifferentiated if necessary. All sections were then passed through alcohol and xylene and mounted in Canada Balsam.

All mounted sections were viewed under the light microscope.

(B) Identification of Macrophages

Treatment of animals with the bacillus Bacille Calmette-Guerin (BCG) is postulated to stimulate the hosts' macrophages. Mice were vaccinated with BCG to study whether an increase in the macrophage population in the liver (the site where control of tetrathyridial multiplication occurs, as indicated in exp. 2) will result in a difference in the rate of tetrathyridial proliferation. Infected liver pieces from BCG treated and control (saline treated) mice were stained with Hematoxylin and Eosin, and Carmalum stains, the latter being a counter stain for trypan blue (ingested by the macrophages) which has been administered subcutaneously to the mice during the infection period. The tissues to be examined were fixed in Bouin's, blocked and sectioned as described above. The staining with Hematoxylin and Eosin was carried out as outlined above, and the staining with the Carmalum stain was carried out as follows.

Carmalum Stain:

The sections were dewaxed, passed through alcohol, washed in tap water and stained in Ravitz's Carmalum (20 g ammonium alum + 150 ml distilled water + 150 ml glycerine + 2 g carminic acid) for 15 minutes. The sections were then rinsed in water, dehydrated and mounted in Canada balsam.

(C) Preparation and Staining of Peritoneal Cell Smears

Transfer of peritoneal cells into irradiated mice were found to depress the rate of multiplication of the parasite (Exp. 2 - Effect of cellular transplants on tetrathyridial multiplication). This initiated a study of the

peritoneal cell population and of the cellular changes occurring with time within the population due to the proliferation of the parasite. Peritoneal cell smears from mice of different infection periods were made and these were then stained with Leishman and Giemsa and then viewed in oil immersion under a light microscope.

a) Peritoneal Cell Smears:

Peritoneal cells were obtained and counted as previously explained. The suspension was recentrifuged at 350 g and a part of the cell pellet was picked up on a wooden stick and dispersed in a small drop of foetal calf serum (FCS; CSL, Melbourne) on a microscope slide. A smear was then made using the edge of another slide. It was air dried, fixed in methanol and dried again.

b) Staining of Peritoneal Cell Smears:

i) The fixed smear was washed with tap water to lyse any red cells present.
ii) The smear cells were stained with 1 volume of Leishman stain (prepared from Leishman powder; BDH) for 3 minutes.
iii) The stain on the smear was diluted with 2 volumes of 0.4% $NaHCO_3$ solution for 10 minutes and then washed with tap water
iv) Five ml of Giemsa stain (BDH) were diluted with 40 ml of 0.4% $NaHCO_3$ solution and filtered. Two volumes of this stain were left on the smear for 30 minutes.
v) The smears were washed in tap water, dried and examined.

Production of burnt lesions in the livers of mice:

When mouse liver tissue is burnt, an inflammatory reaction occurs, and regeneration of tissue follows. This is accompanied by collagen formation. A small lesion made with a cautery knife was used to study the ability of the athymic mouse to produce collagen in wound healing.

Mice were anaesthetized and a small incision was made in the skin and abdominal wall on the left side of the ventral surface just under the rib cage to expose the liver. Using a

cautery knife, a small piece of liver tissue (about 0.1 sq cm) was burnt and the abdominal wall and skin sewn up. The wound was swabbed with sulphonamide to prevent bacterial infection. (I wish to thank Dr. P. Janseen, Zoology Department, ANU, for demonstrating and helping me with the procedure). The mice were placed in a clean cage and kept in a room away from all other mice.

EXPERIMENT 1

THE ROLE OF THE T-LYMPHOCYTE ON TETRATHYRIDIAL MULTIPLICATION IN MICE

Two functionally distinct classes of lymphocytes are involved in vertebrate immunity; the B-lymphocytes and the T-lymphocytes. The B-cells derive their name from their derivation in birds from the Bursa of Fabricius. Their primary focus in mammals is less certain, but they can be obtained from the bone marrow. The thymus is the site for T-cell production in all vertebrates. The function of the B-cell is more clearly understood. When stimulated by a specific foreign antigen they proliferate and some of the cells become transformed into plasma cells, which secrete antibody. With many types of antigen, B-lymphocytes require 'help' from antigen stimulated T-cells in order to respond to an antigen; thus both classes of lymphocytes are necessary for the production of many antibodies. Besides acting as 'helper' cells to B-cells, T-cells have other functions when stimulated by an antigen. They may in certain instances, suppress B-cell activity; or they may initiate the complex reactions giving rise to the delayed type of hypersensitivity reaction; or they may be involved in the allograft rejection.

Antibody production against M. corti in mice, as demonstrated by Kowalski and Thorson (1972a), implies a B-cell response. The role of T-cells in this infection has not been published but was reported by me in my preliminary studies (Pollacco, 1975) in which I experimented with athymic mice. Tetrathyridia were found to multiply at a much faster rate in athymic mice than in normal mice of the same inbred strain. A thymus cell graft restored the athymic mouse to normal. T-cells are therefore important in limiting tetrathyridial proliferation in the normal mouse. As shown from Kazacos (1976) work, and as indicated in this thesis (experiment 2; cellular transplants to intact, irradiated and athymic mice), immunity to tetrathyridia acts in the liver. From this it follows that the site of T-cell action in limiting the parasite mutiplication is in all probability

the liver. Specht and Widmer (1972) carried out a detailed study on the response of the mouse liver to M. corti. Within the first 10 days of infection, the lesions formed by the migrating parasite become completely filled with many neutrophils and moderate numbers of macrophages. Adjacent to the lesions, a broad zone of hepatocyte necrosis mingled with an inflammatory infiltrate developed. Collagen formation was evident on the 13th day post infection, and fibrosis became maximal by the 20th day. By this time, a diffuse infiltrate of neutrophils, macrophages and fibroblasts was observed. Tetrathyridia were seen scattered in fibrous tissue, surrounded by epitheloid cells, but their encapsulation was incomplete.

The mouse's reaction to the parasite during the first 20 days of infection can be separated into a cellular and a fibrocytic response (Specht and Widmer, 1972). A comparative study of the cellular and fibrocytic response in the livers of infected normal and athymic mice could perhaps indicate the role of the T-cell in limiting tetrathyridial multiplication. For such a study, female BALB/c normal, athymic and athymic mice reconstituted with T-cells (see Methods P.6) were infected with 30 tetrathyridia. Two mice from each group were killed on days 10, 15, and 20 post infection. Small pieces of liver from each mouse were fixed in Carnoy's fluid and sectioned as described in Methods (P.27). These sections were stained with hematoxylin and eosin to study the cellular reaction, and with either fast green Van Gieson or with picro-Ponceau/hematoxylin, to study collagen formation. The staining procedures were carried out as described in Methods (P.28). All sections were studied under the light microscope.

a) Liver histology of the infected normal mouse

Scattered parasites in normal liver tissue can be seen 10 days post infection. Pustule formation develops, away from the parasite, possibly as an inflammatory reaction to the damage caused by the burrowing tetrathyridia. Leucocytes, mainly neutrophils, with some macrophages and a few lymphocytes, were present in the pustules (see Plates 1 and 2). Fifteen

Plate 1 Normal mouse liver, 10 days post infection, showing a tetrathridium and a tetrathyridial burrow containing a copious cellular infiltration; stained with hematoxylin and eosin. x 276

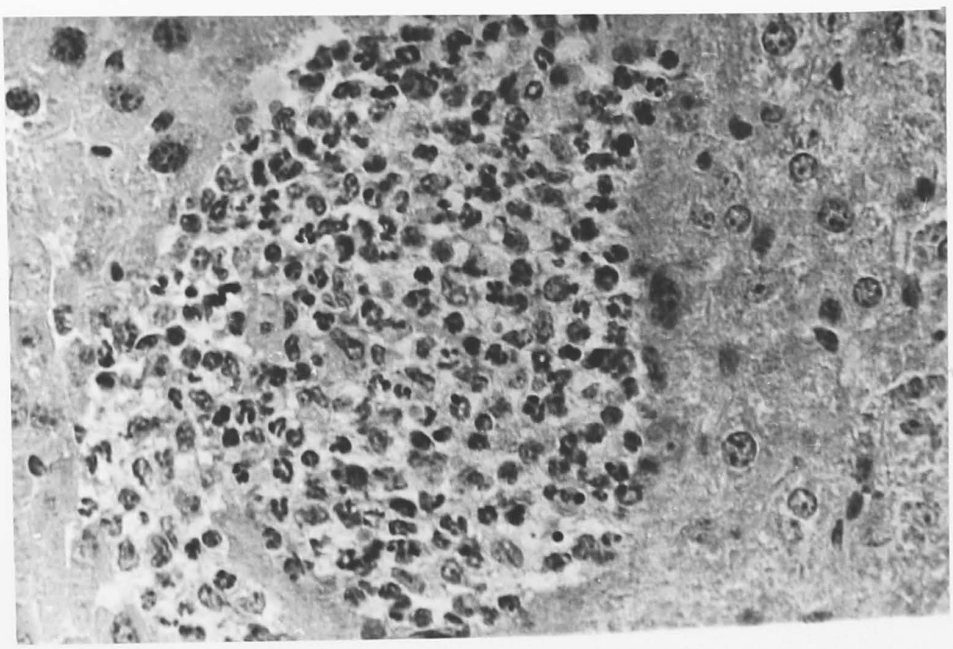


Plate 2 High power view of the cellular infiltration in the tetrathyridial burrow shown in Plate 1. x 1728

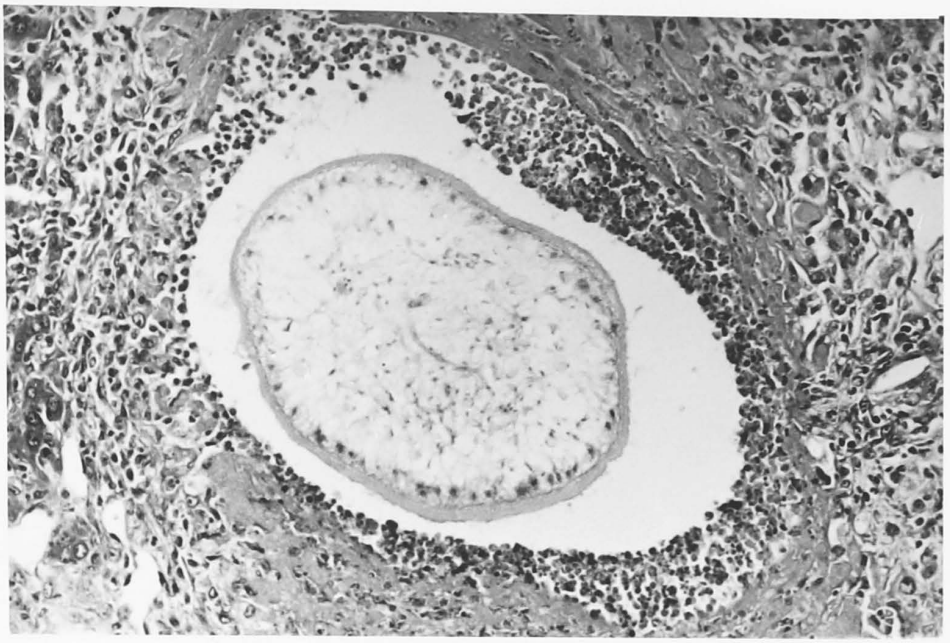


Plate 3 Normal mouse liver, 15 days post infection, showing a tetrathyridium surrounded by a cellular infiltration and collagen fibres; stained with hematoxylin and eosin. x 690.

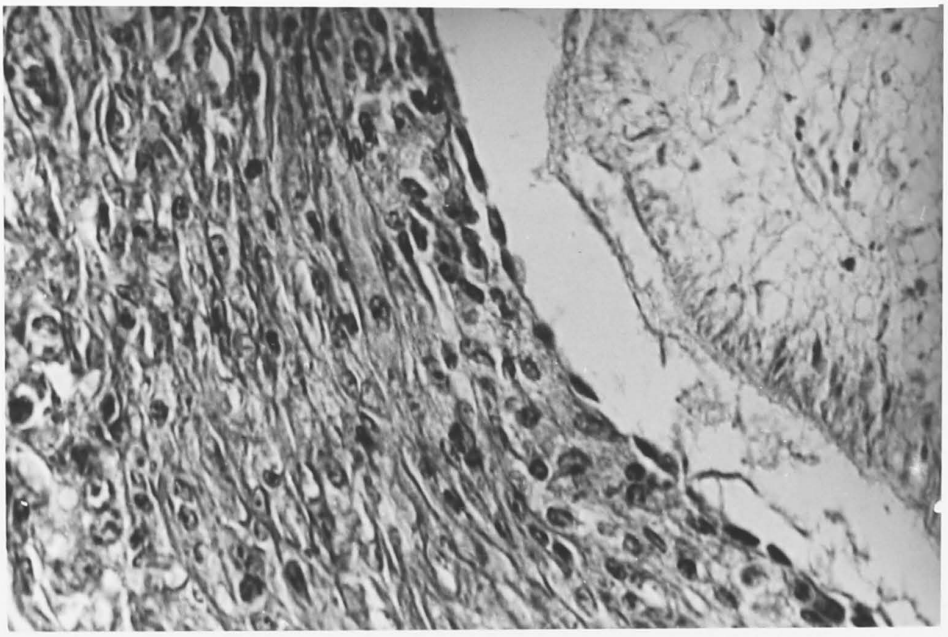


Plate 4 Collagen fibres forming around a tetrathyridium present in normal mouse liver, 15 days post infection; stained with picro-Ponceau/hematoxylin. x 1728

Plate 5 Normal mouse liver, 20 days post infection, showing cellular infiltration and rings of collagen fibres forming around the parasite; stained with hematoxylin and eosin. x 276

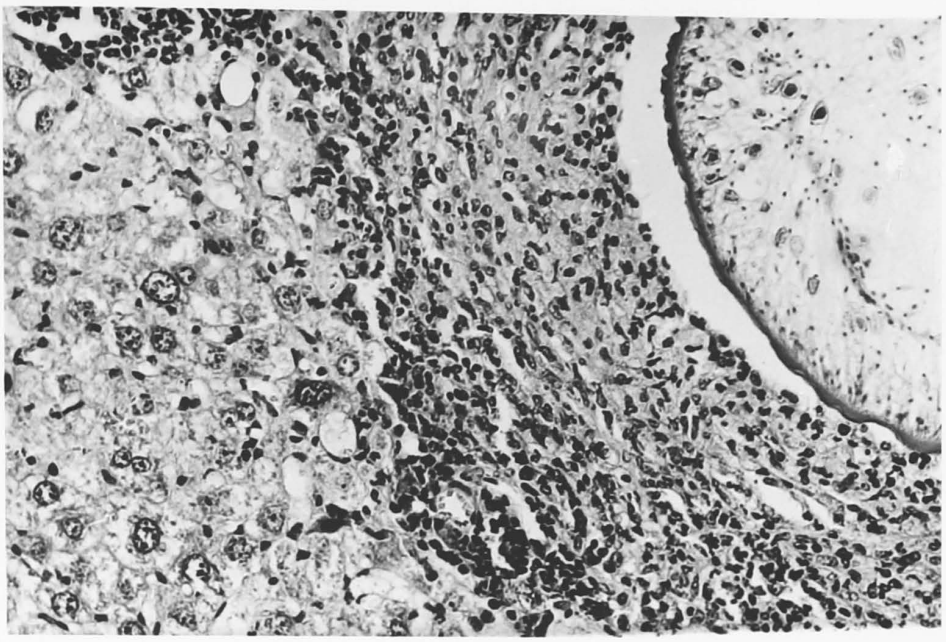


Plate 6 High power view of the cellular infiltration and collagen fibres forming around the tetrathyridium shown in Plate 5. x 690.

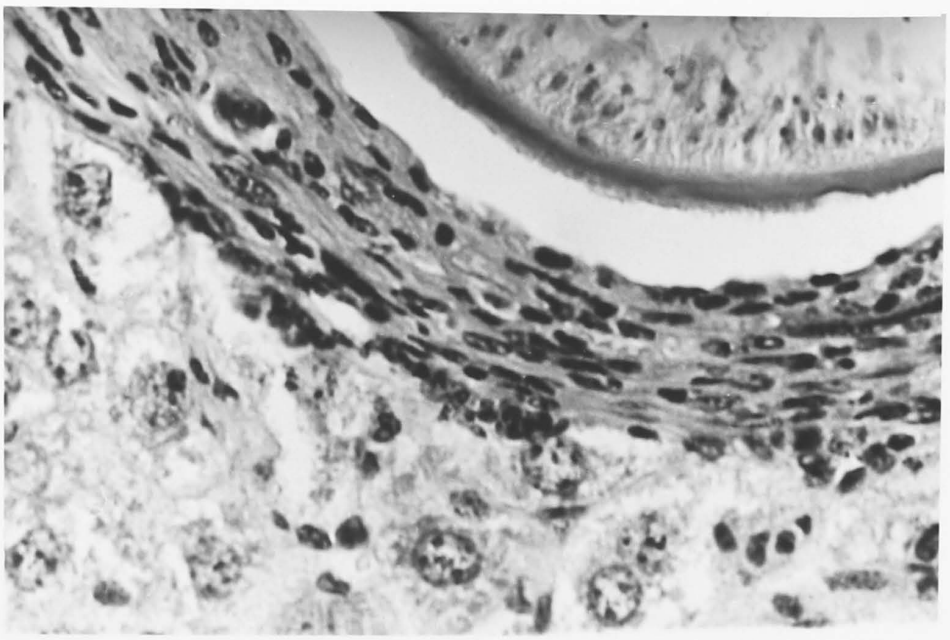


Plate 7 High power view of the collagen fibres forming around the tetrathyridium shown in Plate 5. x 1728.

days post infection collagen formation was detectable in inflammed areas and aggregations of granulocytes together with some macrophages, were present surrounding the parasite (see Plates 3 and 4). The liver became highly infiltrated with collagenous fibres, neutrophils, macrophages and fibroblasts on day 20 post infection (see Plates 5, 6 and 7). However only parasites apparently already dead when fixed were found totally encapsulated.

b) Liver histology of the infected athymic mouse

Parasites were found in the liver tissue on days 10 and 15 post infection with a few granulocytes surrounding the tetrathyridia (see Plates 8 to 11). The numbers of tetrathyridia infecting the liver increased greatly by day 20 post infection, and a few burrows containing an inflammatory reaction were seen. Some granulocytes and macrophages surrounded the tetrathyridia, but the liver, where it had not been destroyed by the parasite, appeared normal in histological structure (see Plates 12 to 15). Red blood cells were present in some lesions. Fibrogenesis and encapsulation of the parasite in fibrous tissue were never observed.

c) Liver histology of the infected T-cell reconstituted athymic mouse

Athymic mice reconstituted with T-cells, (see Methods P.6) produced both a cellular and a fibrocitic response against the penetrating tetrathyridia. Collagen formation was detected 15 days post infection, and the liver became infiltrated with leucocytes, mainly neutrophils, with some macrophages and a few lymphocytes (see Plate 16). The inflammatory reaction had increased intensely by day 20 post infection and incomplete collagenous capsules were formed around the parasites (see Plate 17).

d) Liver histology of the infected athymic mouse grafted with spleen cells

Four groups of female athymic BALB/c mice (age 8 weeks) received spleen cell grafts, intraperitoneally, from normal

Plate 8 Athymic mouse liver, 10 days post infection, showing a tetrathyridium surrounded by a slight cellular infiltration; stained with picro-Ponceau/hematoxylin. x 690.

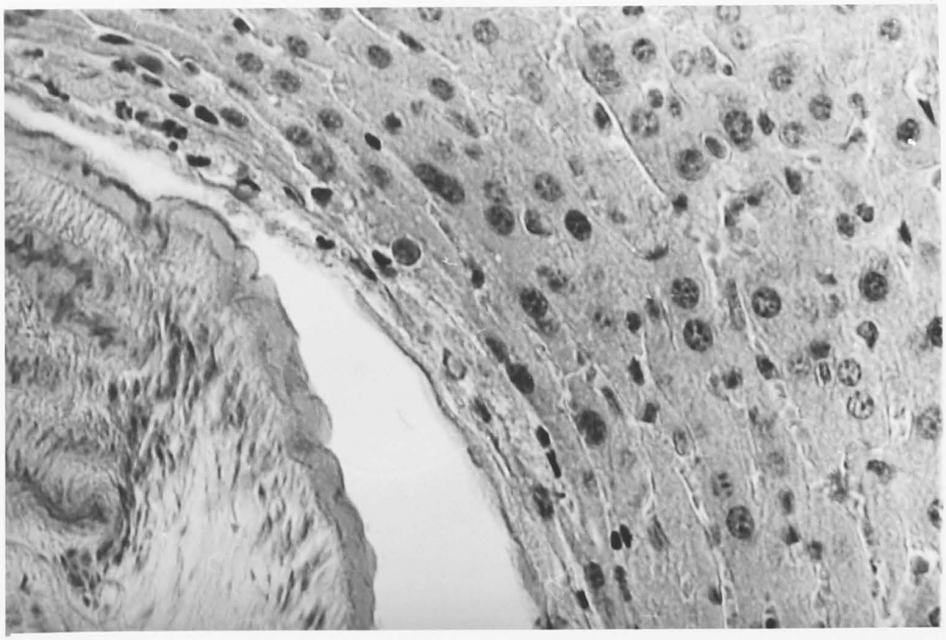


Plate 9 High power view of the tetrathyridium and adjacent hepatocytes shown in Plate 8. x 1728.

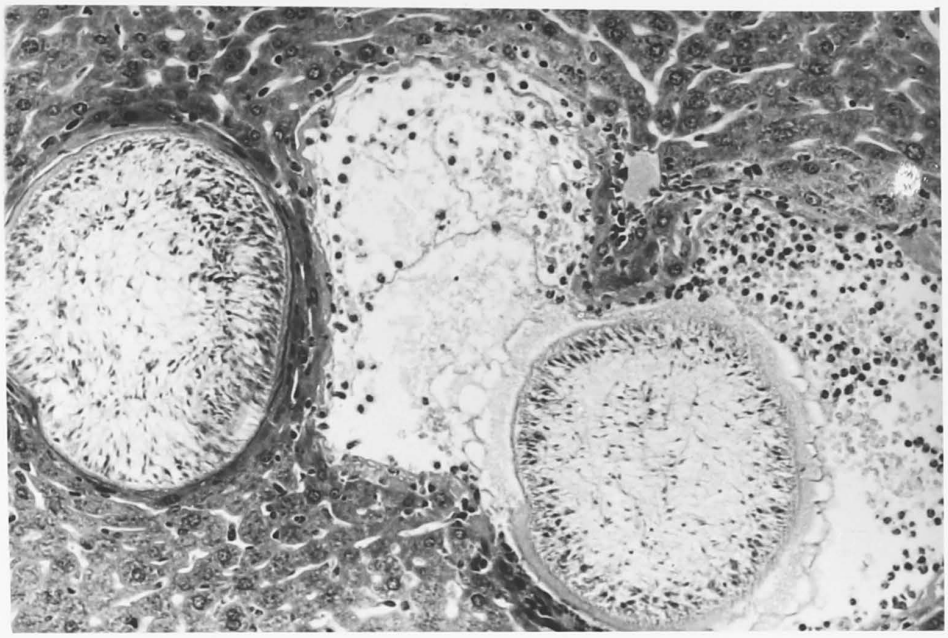


Plate 10 Athymic mouse liver, 15 days post infection, showing tetrathyridia and tetrathyridial burrows containing some cellular infiltration and red blood cells; stained with picro-Ponceau/hematoxylin. x 690.

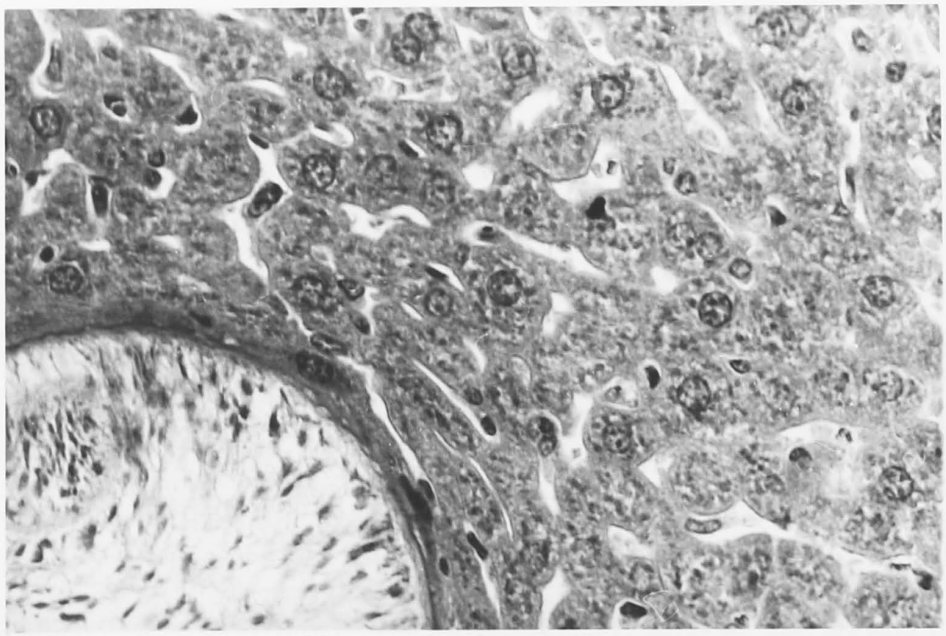


Plate 11 High power view of liver hepatocytes adjacent to a tetrathyridium shown in Plate 10. x 1728.

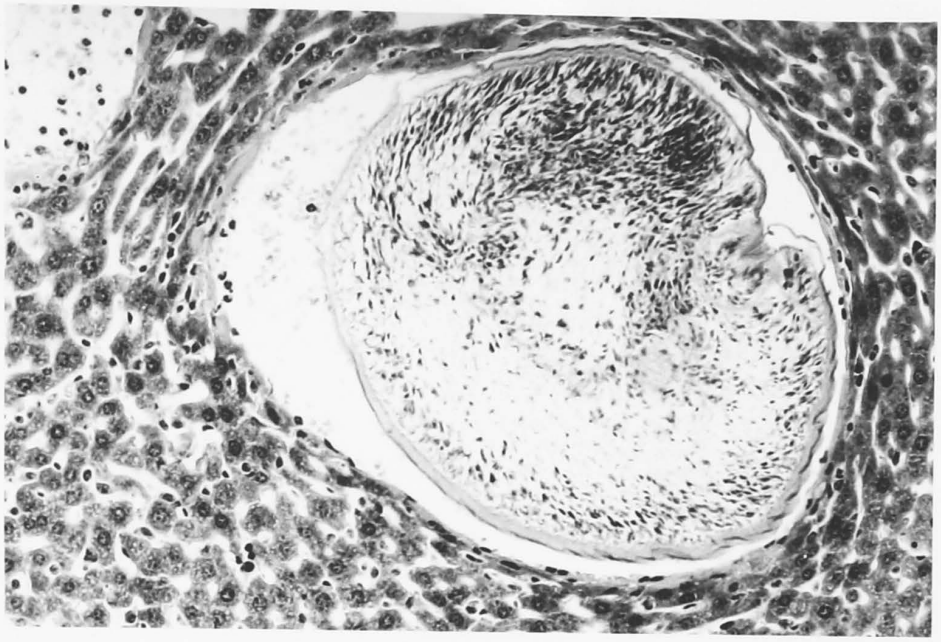


Plate 12 Athymic mouse liver, 20 days post infection, showing a tetrathyridium surrounded by liver cells; stained with hematoxylin and eosin. x 690.

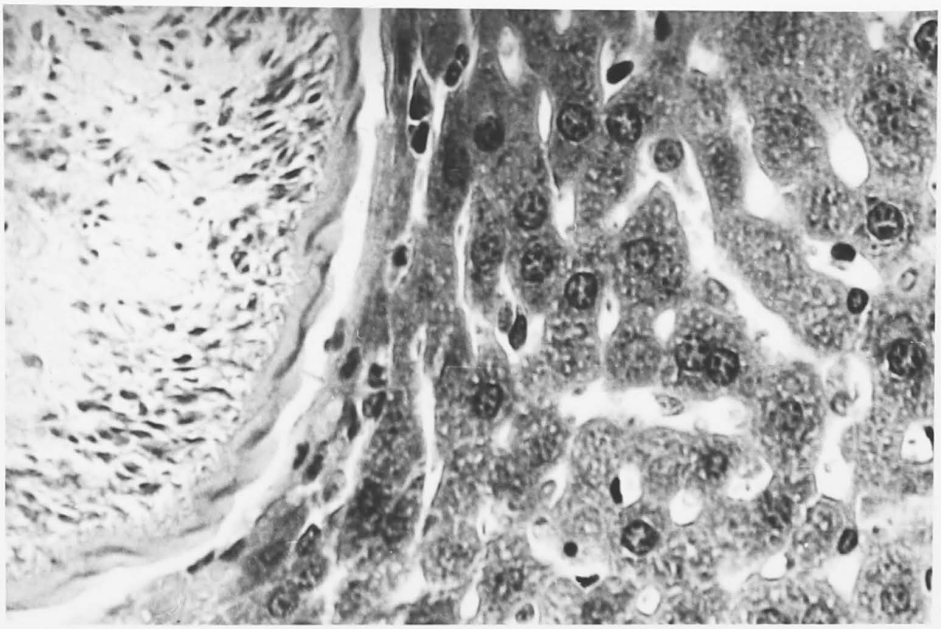


Plate 13 High power view of the liver cells adjacent to the tetrathyridium shown in Plate 12. x 1728.

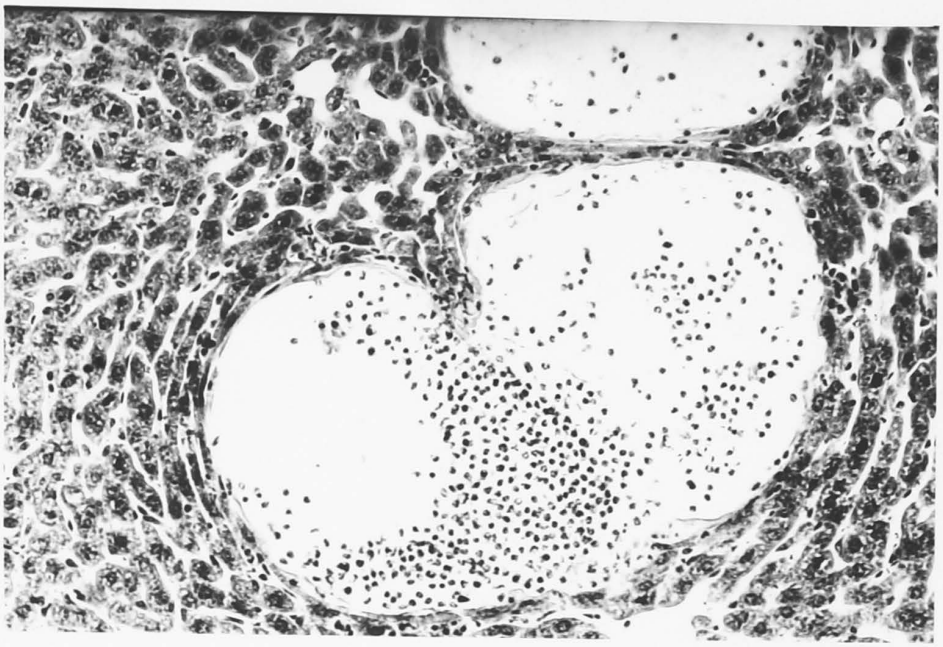


Plate 14 A tetrathyridial burrow containing some cellular infiltration present in athymic mouse liver infected for 20 days; stained with hematoxylin and eosin. x 690.

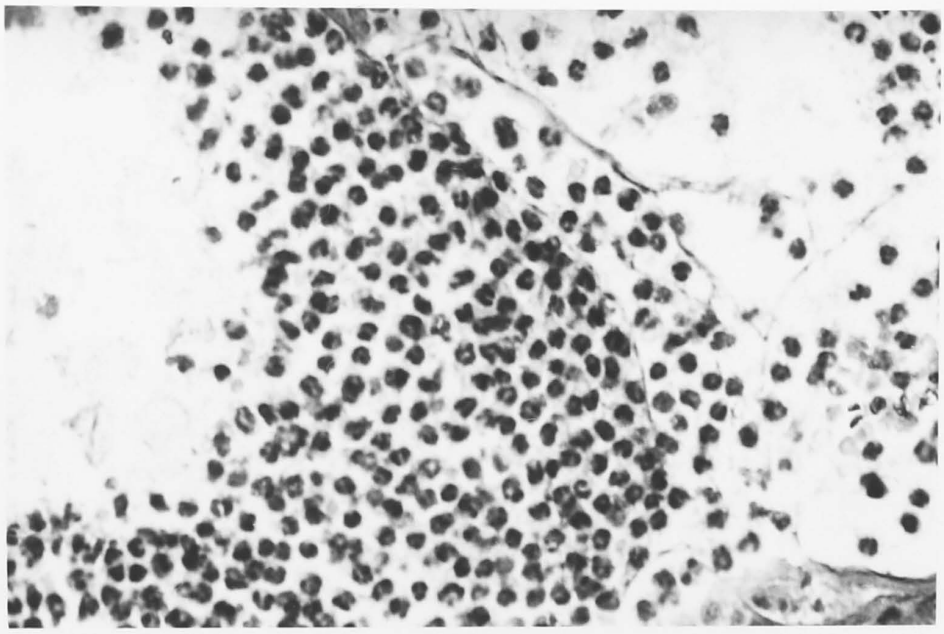


Plate 15 A high power view of the cellular infiltration seen in Plate 14. x 1728.

Plate 16 Liver, 15 days post infection, of the athymic mouse reconstituted with thymus cells containing a tetrathyridium surrounded by a cellular infiltration and collagen fibres; stained with hematoxylin and eosin. x 690.

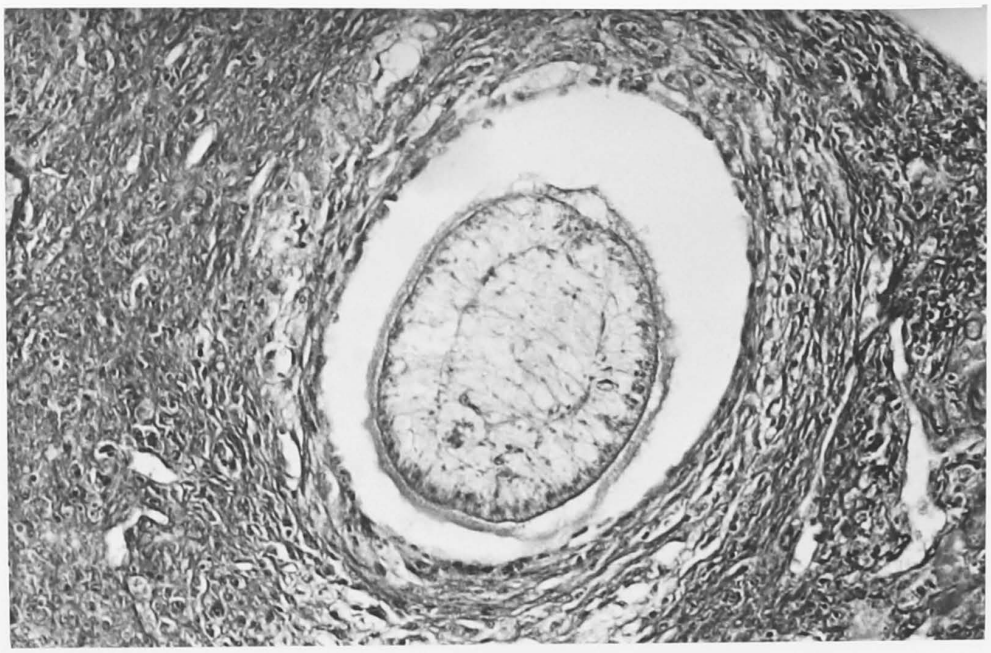


Plate 17 Liver, 20 days post infection, of the athymic mouse reconstituted with thymus cells containing a tetrathyridium surrounded by collagen fibres; stained with fast green Van Gieson. x 690.

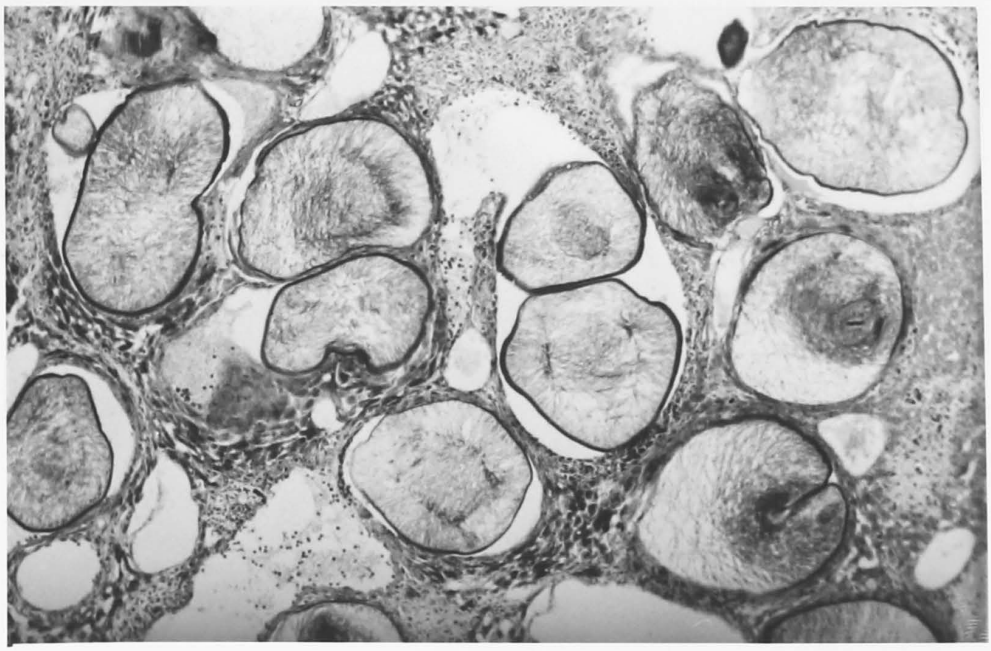


Plate 18 Tetrathyridia, 20 days post infection, seen in athymic mouse liver receiving no spleen cell graft. A slight cellular infiltration is seen; stained with fast green Van Gieson. x 1728.

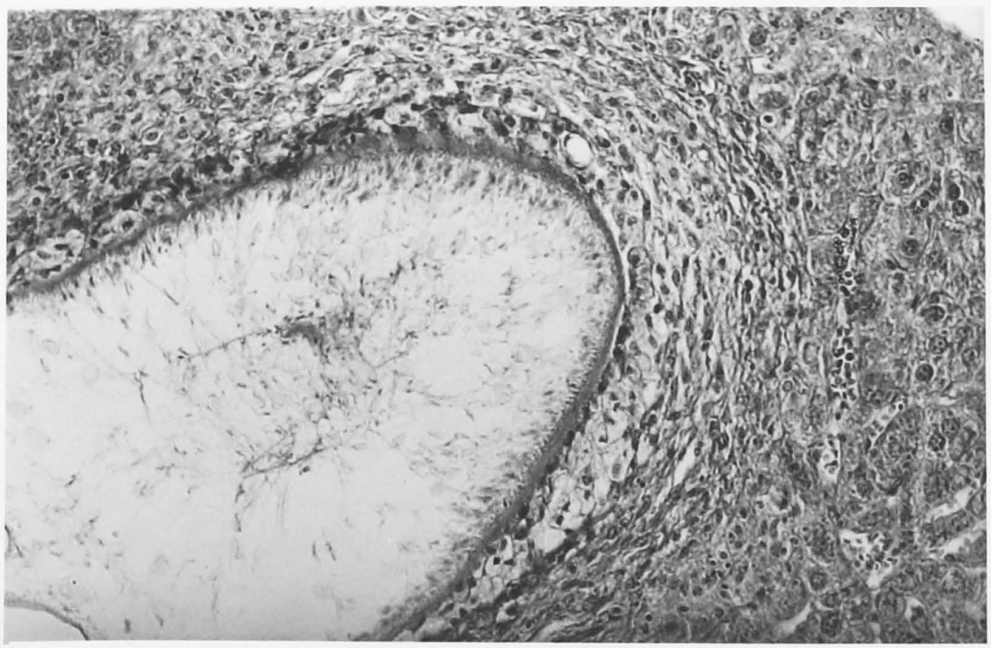


Plate 19 A tetrathyridium in the liver, 20 days post infection, of an athymic mouse receiving a spleen cell graft from a non infected normal mouse. A cellular infiltration and collagen fibres are seen surrounding the parasite; stained with fast green Van Gieson. x 690.

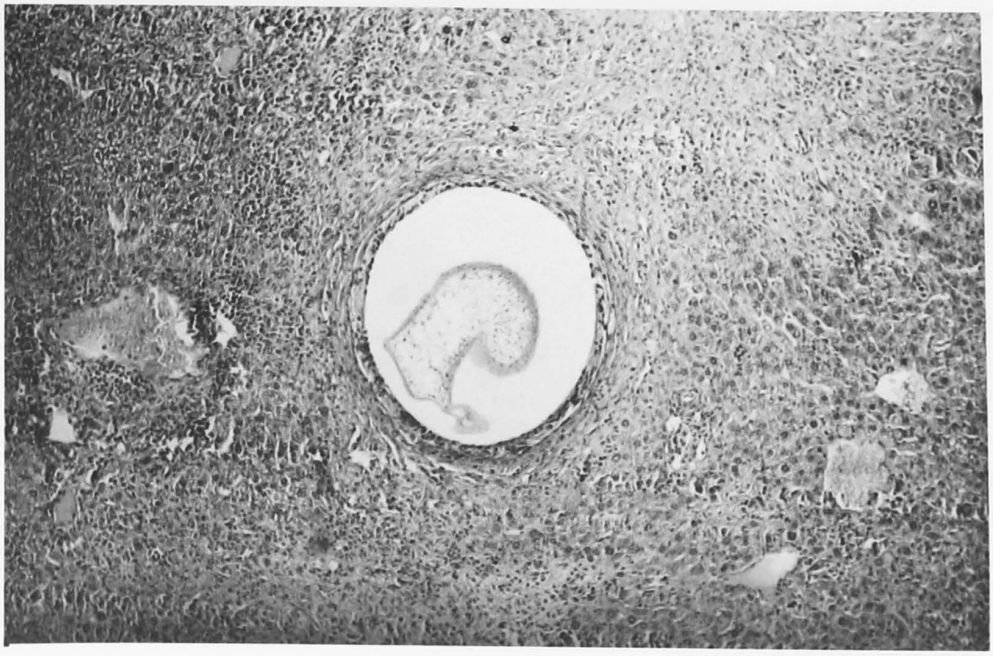


Plate 20 A cellular and fibrocitic reaction to tetrathyridia, 20 days post infection, in the liver of an athymic mouse receiving a spleen cell graft from 1 week infected normal mouse; stained with hematoxylin and eosin. x 276

Plate 21 High power view of a tetrathyridium surrounded by a cellular infiltration and collagen fibres, shown in Plate 20. x 690.

TABLE 1 Liver Histopathology of the Infected Mouse

Type of Infected Mouse	Day of infection	Collagen		Cellular Infiltration
		Inlesion	Round parasite	
Normal	10	-	-	+
	15	+	+	++
	20	+++	++	+++
Athymic	10	-	-	+
	15	-	-	+
	20	-	-	+
Athymic + thymus graft	10	-	-	+
	15	+	+	++
	20	+++	++	+++
Athymic + spleen cell graft from:				
a) infected donor	20	+++	++	+++
b) uninfected donor	20	+++	++	+++

BALB/c mice, which had been infected with 100 tetrathyridia on weeks 5, 3 and 1 and from uninfected mice respectively. The recipient mice were infected with 100 tetrathyridia and were killed 20 days later (see exp. 2, section C). Pieces of their livers were fixed in Bouin's fixative and then blocked, sectioned and stained with Van Gieson as explained in Methods (P.28). The sections were examined under the light microscope and an inflammatory reaction with collagen fromation was present in all groups of mice, except the ones receiving no spleen cell grafts, irrespective of whether the spleen cells came from infected or non infected mice (see Plates 18 to 21). (All the results are summed up in Table 1).

Collagen formation by the athymic mouse

The athymic mouse does not produce collagen fibres in the liver in response to the invading tetrathyridia. However, it is not known whether a mouse lacking T-cells can produce collagen in the liver, (indeed it is present around blood vessels), in other circumstances, or whether it lacks the appropriate immunological stimulus to trigger collagen formation. To find whether the athymic mouse can produce collagen in a non immunological lesion, the following experiment was carried out.

A small area of liver tissue was burnt with a cautery knife (as described in Methods, P.30) in 10 normal and in 10 athymic BALB/c female mice (age 8 weeks). These mice were killed 10 and 15 days post treatment and the area of liver containing the burnt lesion was fixed in Carnoy's fixative. Sections stained with picro-Ponceau were compared under the light microscope and they all showed an extensive inflammatory reaction in the burn with collagen deposition.

Conclusions

Thymus derived lymphocytes (T-cells) are important in limiting tetrathyridial multiplication. This seem to be achieved by

a) collagen formation, which possibley acts by physically restricting the movement and growth of the parasite
b) an extensive cellular reaction, involving mainly neutrophils,

macrophages, and some lymphocytes. Specht and Widmer (1972) believe that this inflammation is a nonspecific reaction, independent of the parasite, to the disruption of the blood supply to the liver parenchyma caused by the burrowing tetrathyridia. This reaction may perhaps also act in limiting the parasites' movement and growth by producing severe alterations in the liver as a parasite substrate.

In the absence of T-cells, the infected liver retains its normal structure, with very little evidence of damage to the hepatocytes. Few and limited areas of cellular infiltration occur away from and adjacent to the parasites. However, in the presence of T-cells the liver shows extensive cellular infiltration with collagen deposition. T-cells do not seem to be involved with tissue repair since liver lesions caused by burning heal in both normal and the athymic mouse. This indicates that the collagenous inflammatory reaction, which is never observed in infected athymic mice, is possibly initiated via the excretory and secretory products of the parasite, which stimulate T-cells to trigger such a collagenous inflammatory reaction to limit the parasites' movement and growth.

Spleen cell grafts from either infected or non infected donors, restores the capacity of T-cell deficient mice to respond to invading tetrathyridia with an extensive cellular reaction and collagen formation in the liver. The spleen cell graft contains lymphocytes, presumably both T- and B-lymphocytes, but, since a thymus graft produces a similar effect, probably it depends for its effect on the T-cell population of the spleen.

EXPERIMENT 2

CELLULAR TRANSPLANTS TO INTACT, IRRADIATED AND ATHYMIC MICE

Irradiated mice have been extensively used in the field of cellular immunology. On irradiating mice with a lethal dose of radiation (which varies with the strain of mice used), the immune system of the mouse is almost completely inactivated. Hence by grafting different cellular populations from normal donor mice to irradiated mice of same inbred strain, one can study the role played by different cell populations. Kelly, Dineen and Love (1973) irradiated rats to study the capacity of sensitized lymphocytes to expel damaged nematode parasites (Nippostrongylus brasiliensis). Bone marrow cells were transplanted into irradiated mice (Neas, Friedberg, Self, 1966) to study their effect on Hymenolepis nana.

The thymus, as the source of T-cells, has been removed from various experimental animals by workers who wished to study the role of T-cells in parasitic infections. Bindseil (1971) showed that thymus cells are important in checking infections in mice with Ascaris suum, since thymectomized animals harboured more parasites than non thymectomized controls. However research into the complex mechanics of immunology have brought together a specially bred mouse which genetically lacks both thymus and hair (thus referred to as 'nude' mouse). The 'nude' mouse is also being used as a means to study the role of T- and B-cells in parasite infections. Hsu and Hsu (1976) carried out a study on the immunopathology of schistosomiasis in athymic (nude) mice.

I have used both irradiated and athymic mice in my experiments to study the role of cells in mice infected with tetrathyridia of M. corti. Some cell transfers to normal mice were also carried out for comparison with transfers to irradiated and athymic mice. All cell transfers were carried between mice of the same inbred strain to avoid host versus graft reaction.

A. Transplants in the Normal Mouse

i) Transfer of peritoneal cells to normal mice

Peritoneal cells from uninfected normal inbred mice (20 week old, female, CBA/H) and from infected inbred mice (female CBA/H, infected for 12 weeks) were obtained as explained in Methods (P.19). Groups of male inbred CBA/H mice were injected (intraperitoneally) with either normal or "immune" peritoneal cells and were infected immediately afterwards with 20 tetrathyridia. Control mice, receiving no cell transplants, were also similarly infected. All mice were killed 20 days later and tetrathyridia recovered from liver and peritoneal cavity were counted. The results are shown in Table 2 and the statistical analysis of the data is given in the Appendix (P.96).

ii) Transfer of tetrathyridia together with adherent peritoneal cells to normal mice

A CBA/H mouse, infected for 12 weeks, was killed and tetrathyridia were sucked up with a pasteur pipette before washing the peritoneal cavity. These tetrathyridia, when viewed under the light microscope, were totally covered with peritoneal cells. Five male CBA/H mice, 8 weeks old, were each infected with 20 of these unwashed larvae. The tetrathyridia remaining in the peritoneal cavity were washed in saline, and another 5 male CBA/H mice were infected with 20 washed tetrathyridia. All mice were killed 20 days later and the total parasite recovery counted (see Table 3) on which statistical analysis were carried out, (Appendix P.97).

Conclusions

Analysing differences between means by Students T-test has shown that transfer of peritoneal cells from mice infected for 12 weeks with tetrathyridia depressed the rate of tetrathyridial multiplication in mice of the same inbred strain. However no significant difference was obtained when tetrathyridia coated with peritoneal cells were injected into mice.

B. Transplants in Irradiated Mice

CBA/H mice, 8 weeks old, were irradiated with 750 rads

TABLE 2 The effects of transferring peritoneal cells from normal and parasitised mice on the multiplication of tetrathyridia in mice infected immediately after transfer

Donor mice	Number of cells transferred (pooled from several donor mice)	Recipient mice (♂, CBA/H, 8 weeks old) infected with 20 tetrathyridia	Mean number of tetrathyridia recovered per mouse ± standard error, 20 days post infection
Normal	6.5×10^6	5	130.4 ± 21.7
Parasitised	6.5×10^6	5	72.2 ± 10.1*
-	-	5	144.8 ± 31.6

* Using Student's T-test, this mean is significantly different from both the other means at $P < 0.05$ (see Appendix P.96)

TABLE 3 The effects of transferring tetrathyridia together with adherent peritoneal cells on tetrathyridial multiplication in normal mice

Parasite	Recipient mice (♂, CBA/H, 8 weeks old)	Mean number of tetrathyridia recovered per mouse ± standard error 20 days post infection
20 washed tetrathyridia	5	246.2 ± 17.8
20 tetrathyridia plus adherent peritoneal cells	5	178.4 ± 26.2

Using Student's T-test, the means are not significantly different at the 5% level of significance (see Appendix P.97)

of gamma-rays from a ^{60}Co source (see Methods P.23). Different cell populations were obtained (see Methods P.17) from normal 20 week old female CBA/H mice. These cells were injected (intraperitoneally or intravenously) into the irradiated mice, 4 hours after irradiation, which were also given 20 tetrathyridia immediately afterwards. Since the maximum life span of these irradiated mice is 13 days, all mice were killed 10 days post treatment. Three separate experiments were carried out, and results are shown in Table 4 and their statistical analysis described in the Appendix (P.98).

Conclusions

Analysis of variances in Experiment 2i shows that differences are highly significant (see Appendix P.98). Student-Newmar-Keul's (SNK) test (Sokal and Rohlf, 1969) for least significant differences indicates that the transfer of peritoneal cells to irradiated mice gave a significant depression in the multiplication of tetrathyridia compared with normal or irradiated, but unsupplemented mice. The transfer of bone marrow cells, or thymus cells, or both combined, gave significantly higher numbers in irradiated mice than the transfer of peritoneal, lymph node, and spleen cells. While bone marrow cells gave significantly more tetrathyridia than spleen cell transplants, the differences from unsupplemented irradiated or normal mice was not significant. The evidence therefore points to a protective role for peritoneal cell transplantation to irradiated mice, but the biological significance of other transplants are doubtful.

Analysis of variances in Experiment 2ii showed significant differences between the groups of mice. SNK analysis of the least significant differences showed a highly significant increase in tetrathyridial multiplication where thymus cell, or bone marrow plus thymus cells, were transplanted in irradiated mice compared with either irradiated unsupplemented or normal mice. Taken together with Exp. 2i, this result suggests T-cell suppression of the protective response of the mouse. Peritoneal cell transplanted mice gave fewer tetrathyridia than normal, or unsupplemented irradiated mice, but not significantly less.

TABLE 4 The effects of transferring different cell populations to irradiated mice of some inbred strain on the multiplication of tetrathyridia

Mice infected with 20 tetrathyridia	Number of CBA/H mice (♂)			Cells transferred from:-
	Exp 1.	Exp 2.	Exp 3.	
Normal	6	4	5	-
Irradiated	6	5	4	-
Irradiated	7	6	-	Thymus
Irradiated	5	6	-	Bone marrow
Irradiated	7	6	-	Bone marrow plus thymus
Irradiated	6	-	-	Spleen
Irradiated	5	-	-	Lymph node
Irradiated	4	5	4	Peritoneal cells
Irradiated	-	5	4	Peritoneal cells minus macrophages
Irradiated	-	-	4	Peritoneal T-cells
Irradiated	-	-	4	Peritoneal B-cells
Irradiated	-	-	5	Peritoneal B- and T-cells

Donor mice were unparasitised, several of which were killed to supply the required number of cells. Cells were injected intraperitoneally (i.p.) or intravenously (i.v.)

Number and route of cell injection			Mean number of tetrathyridia recovered 10 days post infection ± standard error		
Exp 1.	Exp 2.	Exp 3.	Exp 1.	Exp 2.	Exp 3.
-	-	-	61.5± 8.6	66.75± 8.6	106.8± 7.6
-	-	-	56 ± 5.4	54.6 ± 4	114 ±20
20 x10^6i.v.	45 x10^6i.p.	-	66 ± 9.5	119.5 ±11	-
15 x10^6i.v.	8.6x10^6i.p.	-	78.8±14.3	24.2 ± 7.2	-
35 x10^6i.v.	53.6x10^6i.p.	-	61.6± 5.7	143 18.6	-
213 x10^6i.p.	-	-	36 ± 7.2	-	-
7 x10^6i.p.	-	-	30.6± 5.4	-	-
3.6x10^6i.p.	3.6x10^6i.p.	2.4 x10^6i.p.	21 ± 3.5	37.6 ± 6.4	72 ±14.6
-	0.81x10^6i.p.	0.62x10^6i.p.	-	25.8 ±10.4	80 ± 9.8
-	-	0.13x10^6i.p.	-	-	121.5±13.1
-	-	0.52x10^6i.p.	-	-	65.25±5.7
-	-	0.65x10^6i.p.	-	-	59.2± 5.2

BALB/c

Exp. 2iii again showed significant differences between groups by analysis of variance (Appendix P.105) SNK analysis of least significant difference was carried out. The highest recovery was from irradiated mice given a transplant of peritoneal T-cells, but not significantly greater than normal or unsupplemented irradiated mice. Peritoneal cell transplants, peritoneal B-cell, and peritoneal B-plus T-cells, depressed the recoveries compared with normal, or irradiated, unsupplemented mice. There was no evidence of any effect of depleting the population of macrophages.

The overall conclusion must be that the small numbers of mice together with the high degree of variation in recoveries makes it difficult to draw firm conclusions. However, peritoneal cell transplants do seem to depress multiplication in irradiated mice while macrophages do not seem to show any effect. B-cells from the peritoneal cavity were associated with depression, T-cells with enhancement. T-cell transplants from the thymus were also associated with enhancement. B-cells may provide a basis for the irradiated mouse to mount an antibody response to the parasite, as previously established in the normal mouse. It's possible, but needs to be more firmly substantiated, that T-cells may be associated with suppression of immunity. The varying cell composition of the transplants used in different experiments could well explain the differences between the experiments.

C. Transplants in Athymic Mice

The effects of transferring spleen cells from parasitised and unparasitised mice was investigated. Three groups of BALB/c mice, (7 mice per group) were infected with 100 tetrathyridia. They were killed at 5, 3 and 1 week post infection respectively together with a fourth uninfected group and all their spleens removed and transplanted to athymic mice. The spleen cells (obtained as described in Methods P.17) from each group were pooled and injected into athymic mice as shown in Table 5. All recipient mice were infected with 100 tetrathyridia an hour before cell injection. They were all killed 20 days after infection and the tetrathyridia recovered, counted. Results are shown in Table 6 and their statistical analysis given in the

TABLE 5 Transfer of spleen cells from intact to athymic BALB/c mice

Donor group (7 mice per group, ♀, age 8 weeks)	Recipient group (6 mice per group, ♀, age 8 weeks)	Number of viable spleen cells injected per donor mouse ($\times 10^6$)
A' (parasitised for 5 weeks)	A (athymic)	71
B' (parasitised for 3 weeks)	B (athymic)	43
C' (parasitised for 1 week)	C (athymic)	63
D' (not parasitised)	D (athymic)	31
-	E (athymic)	-
-	F (normal)	-

TABLE 6 The multiplication of tetrathyridia in athymic mice (BALB/c) given spleen cell transplants from normal mice infected for various periods

Group (infected with 100 tetrathyridia)	Number of Mice per Group	Number of tetrathyridia recovered 20 days after infection			Means of the Ratios L/P (+ Range)
		Peritoneal Cavity P	Liver L	Total P+L	
A5WPST	6	160± 46	355± 81	515± 184	2.83(1.15-5.03)
A3WPST	6	181± 81	291± 83	494± 176	2.26(0.85-3.78)
AlWPST	6	217± 50	419± 106	635± 111	3.27(0.55-10.17)
AnST	6	304± 87	404± 102	708± 184	2.07(0.82-4.05)
A	6	668±202	4,351±1009	5018±1176.3	8.01(4.53-11.67)
N	6	323± 70	364± 35	687± 3	2.16(0.76-8.09)

The group of athymic mice receiving no spleen cells has a mean which is significantly different from all the other means (see Appendix P.108)

A - athymic recipient mice
N - normal recipient mice

WP - number of weeks for which donor mice were parasitised

n - normal donor mice
ST - spleen cell transplant

Appendix (P.108).

Conclusions

The numbers of tetrathyridia recovered from the athymic mice not given a spleen cell transplant are much higher than from normal mice or any of the athymic mice given spleen cells. The differences in numbers recovered cannot be analysed by a parametric analysis of variance because the variances of the groups are unequal (Bartlett's test). This arises because group E, the athymic unsupplemented mice, have a higher variance. It is common in biological studies of growth to find that the variance of a measurement is a function of the mean, and that a logarithmic transformation can be used to normalise the variance (Sokal and Rohlf, 1969). A logarithmic transformation satisfied Bartlett's test for equality of variances in this experiment and an analysis of variance was carried out (Appendix P.109). This clearly showed that there were significant differences in the recoveries from the groups. However since the peritoneal populations of tetrathyridia from all the groups did not differ significantly, the differences came from the higher recovery of tetrathyridia from liver of the athymic mice, without spleen cell transplants. The conclusion has been further reinforced by the comparison between each pair of groups by the Mann-Whitney non-parametric test, (Siegel, S., 1956) - only the athymic unsupplemented mice were significantly different.

It is clear that the tetrathyridia multiply much faster in the liver of athymic mice (receiving no transplants) than normal mice. Transplanting spleen cells restores the role in the athymic mouse to that found in the normal mouse. It makes no significant difference whether the spleen cells come from parasitised or unparasitised mice. Differences in the numbers found in the peritoneum are not significant.

EXPERIMENT 3

CHANGES IN THE CELL POPULATION OF THE PERITONEAL CAVITY OF INFECTED MICE

The cellular population of the peritoneal cavity is known to change with each phase of the estrous cycle in the mouse (mcGowan and David, 1969). As mice infected with M. corti accumulate great numbers of tetrathyridia in the peritoneal cavity, the effects of this infection on the peritoneal cell population were studied.

Sixteen female Quachenbusch (Q'busch) mice (age 8 weeks) were infected with 20 tetrathyridia while another 20 female uninfected Q'busch mice were used as controls. On weeks 0, 3, 6, 9 and 12 post infection, 4 mice from each group were killed. The peritoneal cells from each mouse were collected, washed and pooled with those from other mice of the same group and counted, as explained in Methods (P.19). Peritoneal cell smears were made from each group and stained with Leishman and Giemsa stains (see Methods P.30). The smears were studied under oil immersion and a differential cell count was carried out, identifying the cells as follows:

a) Macrophages: Generally large cells with dark staining nucleus and blue cytoplasm with a well defined outline.

b) Lymphocytes: Dense nucleus with clumped chromatin. Slight bluish cytoplasm. Generally only a narrow rim of cytoplasm is present.

c) Neutrophils: Dense lobed nucleus.

d) Mast cells: Cell is covered with dense, very dark, purplish staining basophilic granules.

e) Eosinophils: Nucleus has a closed ring shape.

f) Mesothelial cells: Generally large cells with large pinkish nucleus. Cytoplasm has a faint outline with many projections.

g) Monocytes: Dark nucleus, which appears to be "foamy" due to superimposed lobes. Heavy lines mark the edges of the lobes. The nuclear chromatin is generally diffuse, with light spaces between chromatin strands, giving a coarse linear pattern.

TABLE 7 Changes in the differential peritoneal cell counts over a period of 12 weeks in infected and non-infected mice

	Control mice (uninfected) Number of weeks post infection of the experimental mice					Infected mice Number of weeks post infection			
	0	3	6	9	12	3	6	9	12
Total cell count ($\times 10^6$)	19.6	15.5	19.5	18	25	70	71	40	48
% Cell Counts									
Macrophages	15	12.5	18	11	18	20	4.6	64	42
Activated macrophages	-	-	-	-	-	15	73	0	2.8
Lymphocytes	53	73	64	76	56	5.4	7.6	12	14
Neutrophils	3.4	4	2	3	15	25	1.6	4	18
Mast cells	1.6	0	0	0	0.6	0.5	0	0	0
Eosinophils	1	1.5	2	1	7	28	5.6	9	22
Monocytes	12.8	5	8	9	3.4	1.4	4.6	3	0.6
Mesothelial	13.2	4	6	3	0	2.6	3	8	0.6
Cell Numbers ($\times 10^4$)									
Macrophages	294	193.8	351	198	450	1400	326.6	2560	2016
Activated macrophages	-	-	-	-	-	1050	5183	0	134.4
Lymphocytes	1038	1131	1248	1368	1400	378	539.6	480	672
Neutrophils	66.6	62	39	54	375	1750	113.6	160	864
Mast cells	31.3	0	0	0	15	35	0	0	0
Eosinophils	19.6	23.2	39	18	175	1960	397.6	360	1056
Monocytes	250.9	77.5	156	162	85	98	326.6	120	28.8
Mesothelial	258.7	62	58.5	54	0	182	213	320	28.8

Fig. 1 Changes in infected mice with M. corti of the peritoneal cell population during the first 12 weeks of infection

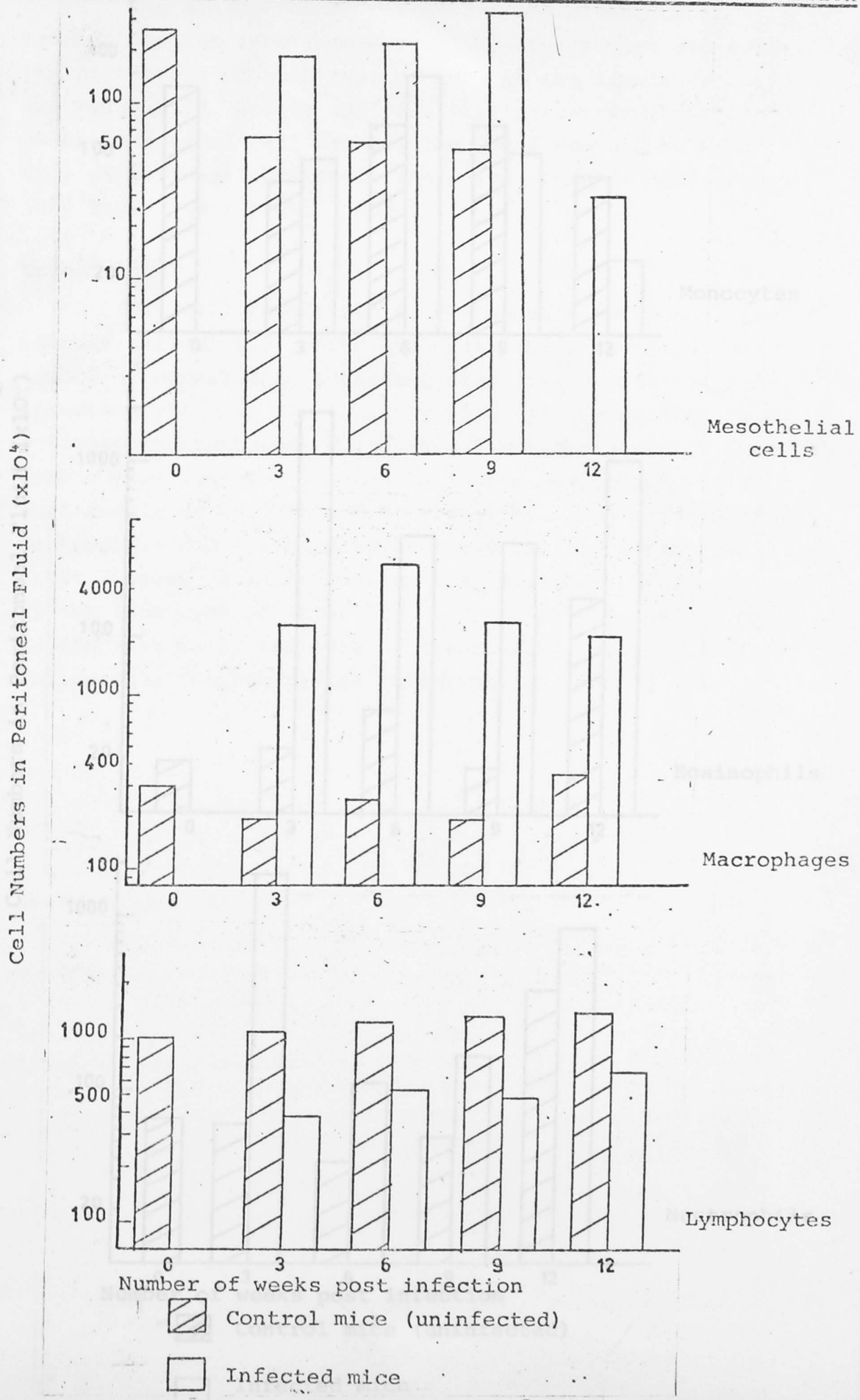
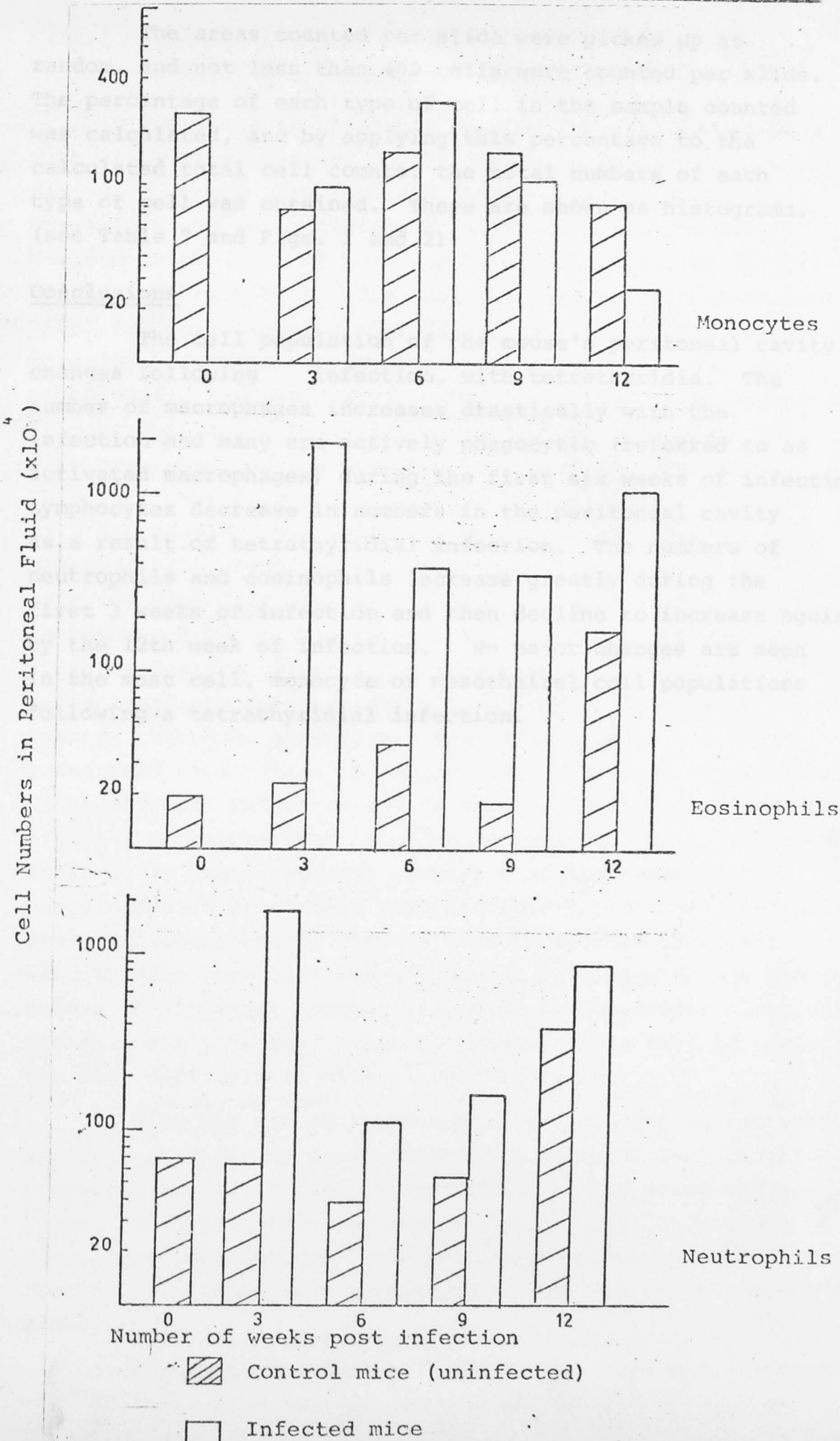


Fig. 2 Changes in infected mice with M. corti of the peritoneal cell population during the first 12 weeks of infection



The areas counted per slide were picked up at random, and not less than 400 cells were counted per slide. The percentage of each type of cell in the sample counted was calculated, and by applying this percentage to the calculated total cell counts, the total numbers of each type of cell was obtained. These are shown as histograms, (see Table 7 and Figs. 1 and 2).

Conclusions

The cell population of the mouse's peritoneal cavity changes following infection with tetrathyridia. The number of macrophages increases drastically with the infection and many are actively phagocytic (referred to as activated macrophages) during the first six weeks of infection. Lymphocytes decrease in numbers in the peritoneal cavity as a result of tetrathyridial infection. The numbers of neutrophils and eosinophils increase greatly during the first 3 weeks of infection and then decline to increase again by the 12th week of infection. No major changes are seen in the mast cell, monocyte or mesothelial cell populations following a tetrathyridial infection.

EXPERIMENT 4

DETECTION OF ANTIBODIES AND ANTIBODY FORMING CELLS IN INFECTED NORMAL AND ATHYMIC MICE

The demonstration that passive immunization of mice with serum from infected donors confers a measure of protection against M. corti (Kowalski and Thorson, 1972a) poses the question as to which classes of antibodies are involved. Various serological methods can be used to test for different classes of antibodies. The indirect haemagglutionation test (IHA) for agglutinating antibodies, has been used to diagnose parasitic diesases e.g. hydatid disease, trichinosis and amoebiasis (Ali Khan, 1974). The Ouchterlony precipitation in agar-gels which tests for precipitating antibodies has also been used for the diagnosis of parasitic infections. This test is very specific and has a considerable potential as a diagnostic tool. So far as cestodes are concerned, Nemeth (1971) demonstrated precipitins in the serum of rabbits infected with Cysticercus pisiformis. Homocytotropic antibodies occur in minute amounts, but their increase is a striking feature of parasitic infections. These antibodies cause immediate hypersensitivity and can be detected by the passive cutaneous anaphylaxis test (PCA), which has found widespread application in immuno diagnosis (e.g. of Ascariasis; Weiszer Isaac, Patterson and Pruzansky; 1968). The fluorescent antibody technique (FA) using whole organisms as a source of antigen, is among the most sensitive of the standard serodiagnostic procedures for parasitic infections. Cerisola, Jose, Rohwedder and Di Corleto (1969) reported excellent results with this test and noted that it always became positive before the indirect haemagglutination or complement fixation tests in early Chagas' disease. However this test is one of the most difficult to attain specificity in.

With the use of these tests, the classes of antibodies produced against the various parasite antigens that can be prepared, and their time of appearance in the serum, can be known. To obtain this information on M. corti infections in mice, these serological tests were carried out on sera obtained from mice infected with tetrathyridia for different lengths of time.

For such experiments 72 CBA/H male mice were infected

with 20 tetrathyridia. Serum was obtained (as explained in Methods P. 9) from 6 mice at weekly intervals for 12 weeks. Uninfected CBA/H, male mice were similarly bled to supply control serum. Athymic and their normal control female BALB/c mice (age 8 weeks) infected with 20 tetrathyridia were killed 3 weeks post infection to supply serum also for these tests. When all the sera were collected, the following tests were carried out.

a) Indirect Haemagglutination Test

This test was used to detect agglutinating antibodies in sera to specific antigens. It depends on the capacity of antibody, when present, to cause agglutination of antigen-coupled SRBC. The technique has been described in the Methods (P. 10). The results of this test are shown in Table 8.

b) Ouchterlony Precipitation in Gel

In this test, serum and antigen diffuse separately through an agar gel towards one another and when the local concentration of the antigen and precipitating antibodies meet at an equivalence point, precipitation bands form, due to antigen-antibody complexes forming.

The preparation of the plates has been described in Methods (P. 11). The 2 antigens, somatic, and excretory and secretory (ES), both with a protein concentration of 6 mg/ml, were each placed in one of the 2 central wells. The sera to be tested were placed in the surrounding wells. The plates were viewed 48 hours later and the number of bands formed scored as shown in Table 9.

c) Passive Cutaneous Anaphylaxis

Passive cutaneous anaphylaxis (PCA) to test for IgE antibodies, was carried out in Wistar rats (male, 15 weeks old) as explained in Methods (P. 13). Sera from uninfected and infected CBA/H mice for 3, 7, and 12 weeks together with sera from athymic and their normal controls, 3 week infected BALB/c mice, were tested. A positive PCA reaction shows up as

TABLE 8 Results of the indirect haemagglutination test on serum from mice infected with 20 tetrathyridia

Strain of mice (age 8 weeks)	Serum from mice infected for: (weeks)	Greatest dilution of serum giving agglutination of antigen fixed SRBC	
		ES antigen	Somatic antigen
CBA/H(♂)	1	1:1	1:3
	2	1:1	1:3
	3	1:1	1:7
	4	1:1	1:7
	5	1:1	1:7
	6	1:3	1:7
	7	1:3	1:7
	8	1:1	1:7
	9	1:1	1:63
	10	1:3	1:63
	11	1:3	1:31
	12	1:3	1:31
	Uninfected	no agglutination	1:1
BALB/c (♀)			
Athymic	3	1:1	1:3
Normal controls	3	1:3	1:7

TABLE 9 Precipitating antibodies detected by gel diffusion in the sera of mice initially infected with 20 tetrathyridia

Strain of mice (age 8 weeks)	Serum from mice infected for: (weeks)	Number of bands forming against the antigens: ES	Somatic
CBA/H(♂)	1	None	None
	2	"	None
	3	"	1
	4	"	1
	5	"	1
	6	"	1
	7	"	1(+1 very faint)
	8	"	1(+1 very faint)
	9	"	2
	10	"	2
	11	"	2
	12	"	3
	Uninfected	"	None
BALB/c (♀)			
Athymic	3	None	None
Normal control	3	None	None

a blue area in the skin of rats. The results obtained from this test were as given in Table 10.

d) Fluorescent Antibody Technique

In this test, sections of the whole parasite were incubated with serum at room temperature. Antibodies present in the serum combine with the specific parasite antigens against which they were raised in the serum of donor animals. The antigen-antibody complexes formed are revealed under the ultraviolet microscope by the addition of an appropriate fluorescent-labelled immunoglobulin.

Sections of tetrathyridia obtained from the peritoneal cavity and from the liver tissue were prepared from athymic and their normal controls, 3 week infected BALB/c female mice, as explained in Methods (P. 14). These sections were incubated in either normal or "immune" serum (obtained from 12 weeks infected ♂ CBA/H mice) and then treated with fluorescent rabbit anti-mouse immunoglobulin (Nutritional Biochemicals) as explained in Methods (P. 16). Controls of untreated sections, or sections treated with only the fluorescent immunoglobulin were also prepared. The results obtained from this test are explained in Table 11.

The Plaque Forming Cell Technique

This technique was demonstrated by Dr Cunningham (JCSMR, ANU). It assays the number of cells secreting antibodies in a particular organ against those parasite antigens which have been isolated by the separation method used and which then conjugate to the SRBC. This test shows directly whether an infected host can respond to its parasite by antibody production. The plaque forming cell technique was carried out to test whether antibody forming cells can be detected in different organs of infected normal and athymic BALB/c mice with M. corti tetrathyridia.

Exp. i Assays of the number of antibody secreting cells, against the tetrathyridial ES antigen in the spleens of uninfected and infected BALB/c mice

In this experiment lymphocytes from the spleens of uninfected and 3 week infected BALB/c female mice infected

TABLE 10 Passive cutaneous analphylaxis test in the rat for IgE antibodies in the sera of mice infected with 20 tetrathyridia

Strain of mice (age 8 weeks)	Serum from mice infected for: (weeks)	PCA reactions using serum at various dilutions against:							
		Somatic antigen				ES antigen			
		Undiluted	1:3	1:9	Control*	Undiluted	1:3	1:9	Control*
CBA/H(♂)	Uninfected**	-				-			
	3	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-
	12	++	+	+	-	+	++	-	-
BALB/c (♀)									
Athymic	3	-	-	-	-	-	-	-	-
Normal control	3	-	-	-	-	-	-	-	-

* Serum heated to 56°C for 30 minutes (IgE relatively heat labile)

** Serum from uninfected mice was used as another control for each test and it always gave negative results

TABLE 11. The indirect immunofluorescent test for the detection of mouse antibody bound to tetrathyridia from the peritoneal cavity and liver of BALB/c mice infected for 3 weeks.

Tissue from 3 week infected BALB/c mice	Preparation of sections	Serum*	Fluorescent Immunoglobulin	Fluorescence on parasite Tegument	Internal
a) Athymic mouse:					
PT+	Frozen	-	-	None	None
PT	Frozen	-	+	None	None
PT	Frozen	Normal	+	None	None
PT	Frozen	'Immune'	+	None	Fluoresces
PT	Wax	-	-	None	None
PT	Wax	-	+	None	None
PT	Wax	Normal	+	None	None
PT	Wax	'Immune'	+	None	Fluoresces
LT++	Wax	-	-	None	None
LT	Wax	-	+	None	None
LT	Wax	Normal	+	None	None
LT	Wax	'Immune'	+	None	Fluoresces
b) Normal control mouse:					
PT	Frozen	-	-	None	None
PT	Frozen	-	+	Fluoresces	None
PT	Frozen	Normal	+	Fluoresces	None
PT	Frozen	'Immune'	+	Fluoresces	Fluoresces
PT	Wax	-	-	None	None
PT	Wax	-	_	Fluoresces	None
PT	Wax	Normal	+	Fluoresces	None
PT	Wax	'Immune'	+	Fluoresces	Fluoresces
LT	Wax	-		None	None
LT	Wax	-	+	None	None
LT	Wax	Normal	+	None	None
LT	Wax	'Immune'	+	None	Fluoresces

+ Tetrathyridia from peritoneal cavity

++ Tetrathyridia in liver

* Normal serum from uninfected CBA/H mice; 'Immune' serum from 12 week infected CBA/H mice

with 30 tetrathyridia, were obtained as described in Methods (P. 17). The excretory and secretory (ES) antigen of the parasite, at a protein concentration of 3 mg/ml, was fixed on to washed sheep red blood cells (SRBC) using 0.01% chromic chloride (explained in Methods, P.25). Either of the 2 lymphocyte suspension in Hanks BSS, together with the antigen attached on to the SRBC, were incubated with complement (guinea-pig serum) in Hanks BSS at 37°C for 55 minutes. Control assays were also carried out using SRBC with no antigen fixed on to them. The number of plaques obtained, which indicate the number of antibody cells against the parasite ES antigen, are recorded in Table 12.

Exp. ii Reverse Plaque Technique

An estimate of the total number of antibody forming cells present in the spleen of normal and infected mice with M. corti was carried out. In this assay the SRBC were attached to rabbit antimouse immunoglobulin (instead of tetrathyridial antigen). The lymphocyte suspension and the immunoglobulin attached SRBC were incubated with the complement and a developer (rabbit antimouse immunoglobulin at a dilution of 1:19 in Hanks BSS). The mode of action of this developer is not known but it is thought to give an estimate from the total number of plaques obtained, of the total antibody secreting cells present in spleens of nonparasitised and parasitised mice (advice given to me by Dr. Cunningham JCSMR, ANU). Results are given in Table 12.

Exp. iii An inhibition test using free ES antigen to show whether the antibody secreting cells detected against the ES antigen fixed SRBC are specific to the ES antigen

A number of assays were carried out to see if the addition of free antigen competes with the antigen fixed on to the SRBC in reacting with the specific antibodies secreted by the lymphocytes.

Spleen cells were obtained from 4 week infected (with 30 tetrathyridia) BALB/c female mice. The ES antigen was

TABLE 12 Assays for the number of antibody secreting cells in the spleens of uninfected and 3 week infected BALB/c mice by the plaque forming cell technique

Antigen attached to SRBC	Number of spleen cells tested from mice: Uninfected	Infected	Number of Plaques Formed	Ratio of antibody secreting cells to spleen cells assayed
ES	156×10^6		279	$1:10^{5.75}$
-	156×10^6		321	$1:10^{5.69}$
ES		340×10^6	32,143	$1:10^{4.02}$
-		340×10^6	2,500	$1:10^{5.13}$
Rabbit antimouse Ig	156×10^6		235,714	$1:10^{2.82}$
Rabbit antimouse Ig		340×10^6	2,285,714	$1:10^{2.17}$

attached to SRBC as described in Methods (P.25). However in these assays instead of Hanks BSS, different concentrations of free ES antigen ranging from 0 to 60 µg protein in physiological saline were added. Incubation with complement was carried out at 37°C for 55 minutes. The inhibition of plaque formation by increasing concentration of free antigen is shown in Table 13.

As a control for this test, a spleen cell suspension from mice immunized against horse RBC (0.2 mls of 10% horse RBC injected intravenously and killed 5 days later) was prepared. This cell suspension was assayed against horse RBC, using increased concentration of free ES tetrathyridial antigen as above, and incubated with complement as previously described. Any changes, caused by the increase in the concentration of free ES antigen, in the number of plaques formed against horse antigen (see Table 13) were compared with the changes obtained in the number of plaques formed against the ES antigen, as shown in Figure 3.

Exp. iv Development of indirect IgG plaque forming cells against the ES antigen fixed SRBC

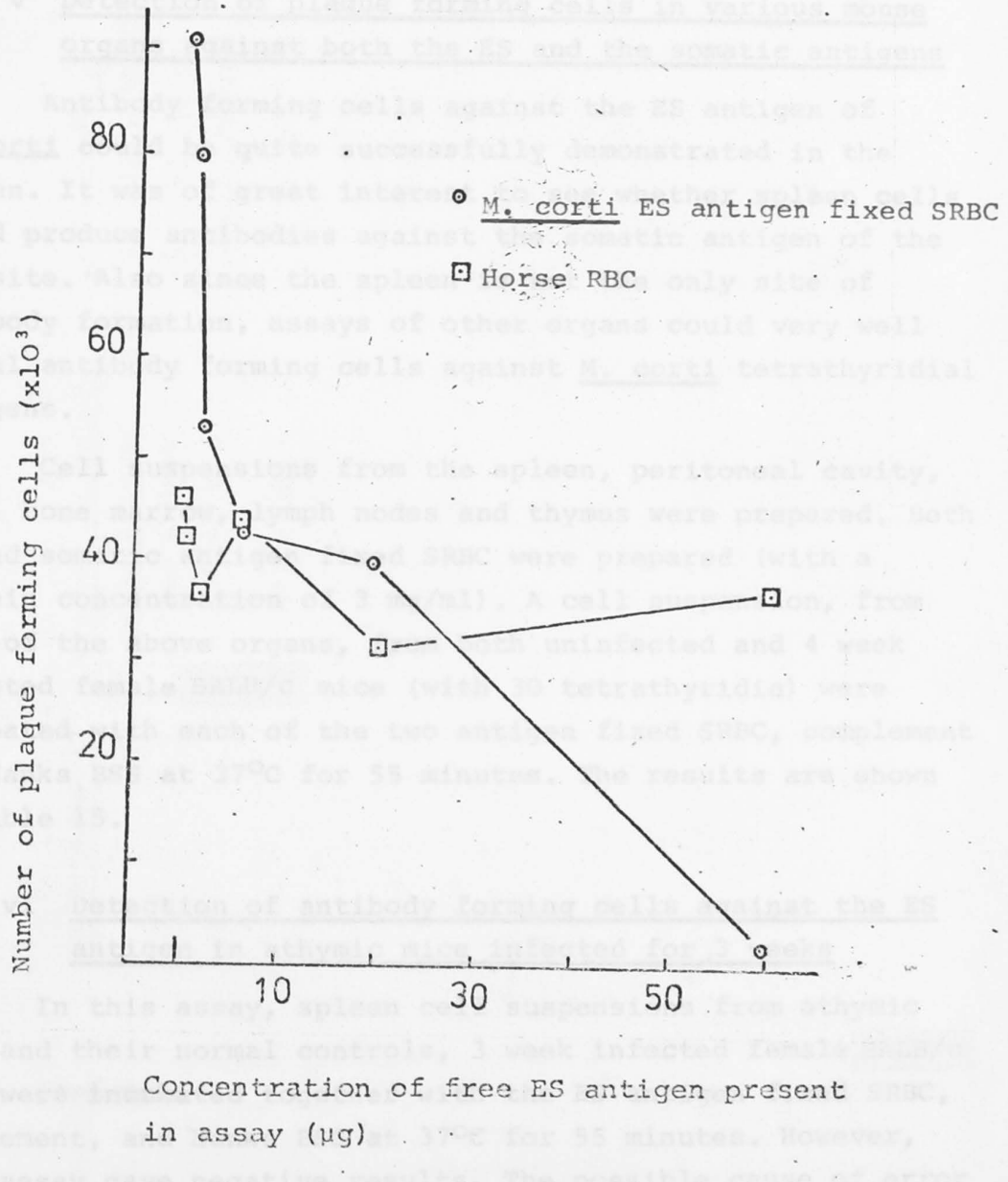
This assay detects any lymphocytes secreting antibodies of the IgG type to the ES tetrathyridial antigens. All the plaques obtained from the previous assays detect lymphocytes secreting antibodies of the IgM type. An IgM molecule has a complex structure (usually a pentamer) and can by itself bind complement. An IgG antibody is a monomer and cannot by itself bind complement. However if rabbit anti-mouse immunoglobulin, (diluted 1:99 with Hanks BSS) as a developer is added, it complexes with IgG antibodies on the surface of lymphocytes and binds complement. Also, this developer inhibits the binding capacity of about 50% of the IgM molecules due to the very large complexes formed, (Cunningham, personal communication) and therefore most of the plaques formed in such an assay are due to secreted IgG antibodies.

In this assay, a spleen lymphocyte suspension from 4 weeks infected female BALB/c mice (with 30 tetrathyridia) together with the ES antigen fixed SRBC, were incubated with

TABLE 13 Competitive inhibition between free ES antigen and ES antigen coupled to SRBC on the binding to ES-specific immunoglobulin on the surface of spleen cells

Antigen used in assay	Concentration of free ES antigen (μg)	Number of spleen cells assayed	Number of plaques formed (x 10^3)
M. corti ES antigen coupled to SRBC:	0	381 x 10^6	93.3
	0.6	"	80
	1.9	"	53.3
	6.0	"	42.6
	19	"	40
	60	"	4
Horse RBC:	0	219 x 10^6	46
	0.6	"	42
	1.9	"	36.6
	6.0	"	44.3
	19	"	31.6
	60	"	37.3

Fig. 1 Competitive inhibition between free ES antigen and ES antigen coupled to SRBC on the binding to ES-specific immunoglobulin on the surface of spleen cells



complement and the developer for 55 minutes at 37°C. The number of plaques formed were estimated, as shown in Table 14. These plaques are referred to as indirect IgG plaques direct IgG plaques can be formed by using specific rabbit anti-mouse IgG . Appropriate controls without developer and/or without antigen fixed SRBC were also assayed.

Exp. v Detection of plaque forming cells in various mouse organs against both the ES and the somatic antigens

Antibody forming cells against the ES antigen of M. corti could be quite successfully demonstrated in the spleen. It was of great interest to see whether spleen cells could produce antibodies against the somatic antigen of the parasite. Also since the spleen is not the only site of antibody formation, assays of other organs could very well reveal antibody forming cells against M. corti tetrathyridial antigens.

Cell suspensions from the spleen, peritoneal cavity, lung, bone marrow, lymph nodes and thymus were prepared. Both ES and somatic antigen fixed SRBC were prepared (with a protein concentration of 3 mg/ml). A cell suspension, from each of the above organs, from both uninfected and 4 week infected female BALB/c mice (with 30 tetrathyridia) were incubated with each of the two antigen fixed SRBC, complement and Hanks BSS at 37°C for 55 minutes. The results are shown in Table 15.

Exp. vi Detection of antibody forming cells against the ES antigen in athymic mice infected for 3 weeks

In this assay, spleen cell suspensions from athymic mice and their normal controls, 3 week infected female BALB/c mice were incubated together with the ES antigen fixed SRBC, complement, and Hanks BSS at 37°C for 55 minutes. However, this assay gave negative results. The possible cause of error was investigated. The chromic chloride was tested and it was found active in binding antigen to RBC. The mice used for all the plaque forming cell assays were BALB/c mice of the inbred strain and therefore their immunological response to M. corti

TABLE 14 Detection of indirect IgG plaque forming cells using rabbit anti-mouse immunoglobulin as the developer

Antigen coupled to SRBC	Developer	Number of spleen cells assayed (from 4 week infected mice)	Number of plaques formed in the spleen cells assayed	Type of antibody forming plaques
ES	-	391 x 10^6	40,357	Mainly IgM
None	-	"	1,071	Mainly IgM
ES	+	"	16,071	IgM, few IgG
None	+	"	714	IgM, few IgG

TABLE 15 The number of plaque forming cells reacting with the ES and somatic antigens of M. corti tetrathyridia in various mouse organs

Organ	Number of cells assayed	Number of plaques formed in the cell suspensions assayed against the fixed SRBC with:-		
		ES antigen	Somatic antigen	No antigen
N* spleen	109 x 10^6	200	50	50
I.** spleen	365 x 10^6	11,429	214	71
N. peritoneal cavity	2 x 10^6	0	0	0
I. peritoneal cavity	7 x 10^6	0	0	0
N. lung	152 x 10^6	0	0	0
I. lung	103 x 10^6	0	0	0
N. bone marrow	17 x 10^6	0	0	0
I. bone marrow	28 x 10^6	0	0	0
N. lymph node	2 x 10^6	0	0	0
I. lymph node	4 x 10^6	60	0	0
N. thymus	103 x 10^6	0	0	0
I. thymus	52 x 10^6	321	7	7

* obtained from uninfected mice

** obtained from 4 week infected mice with 30 tetrathyridia

infections should be the same. The remaining variable was the antigen, and although different samples of the ES antigen may contain the same protein concentration, their antigenic constitution may vary. Perhaps the conditions of culture determine the ES antigen constitution. The short term tetrathyridia are generally more active in culture and their secretory products *in vitro* are probably more similar to the ones secreted *in vivo* in the mouse.

Conclusions

The serological tests have shown that:

a) Due to the low titres of agglutinating antibodies detected against the ES antigen, and during the first 8 weeks of infection, against the somatic antigen, it can only be concluded with certainty that agglutinating antibodies, possibly of the IgM type, are detectable only after the 8th week of infection against the somatic antigen.

b) The Ouchterlony precipitation test gives positive results with a gradual increase in the number of bands with increase in the time of infection against the somatic antigen from the 3rd week of infection. However this test is negative when using serum from 3 week infected athymic mice. This tests detect mainly antibodies of the IgG type.

c) IgE antibodies are detected in the serum obtained from 12 week infected mice. This antibody is produced in mice between the 7th and 12th week of infection.

d) From the fluorescent antibody technique it can be concluded that tetrathyridia obtained from the peritoneal cavity of normal mice are coated with a layer of host immunoglobulin (see Plate 22). This coat is absent in liver tetrathyridia and from tetrathyridia obtained from athymic mice (see Plates 23 and 24). Serum obtained from 12 week infected mice has antibdoes against the internal structure of the parasite suggesting that these antibodies are perhaps produced against the secretions and excretions of the parasite (see Plate 25). No difference could be detected between the results obtained from the frozen and the paraffin sections.

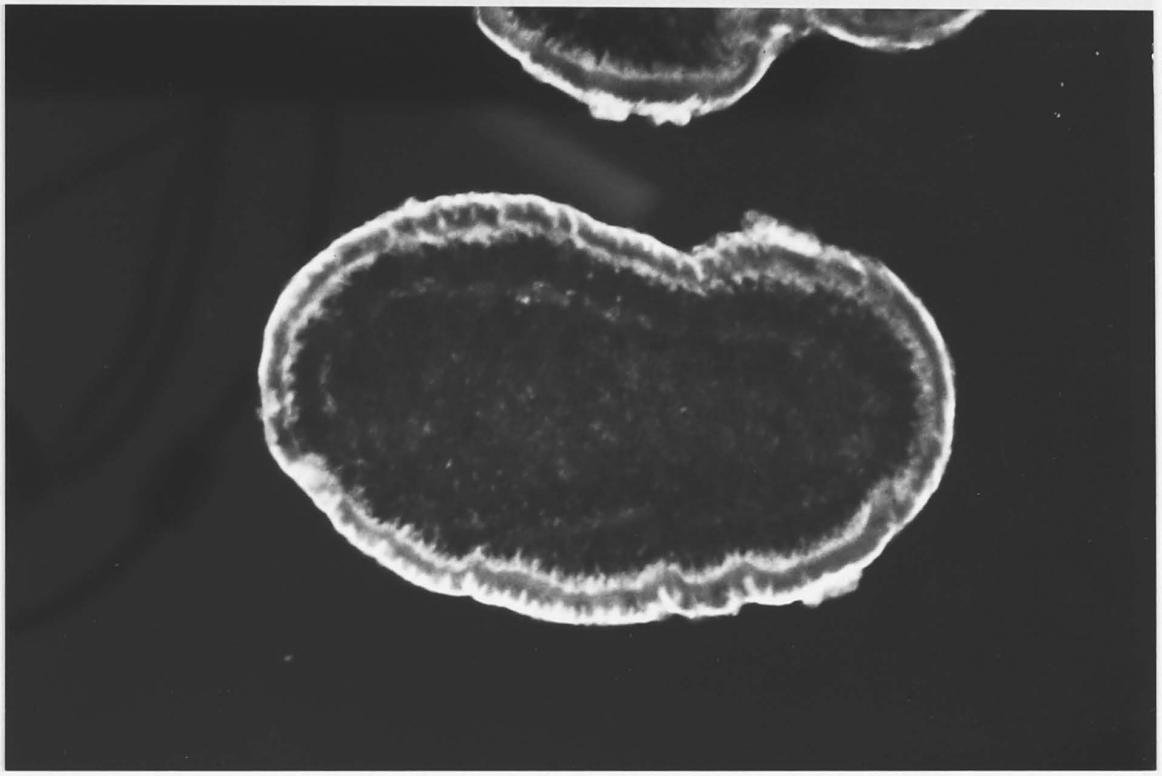


Plate 22 A tetrathyridium from the peritoneal cavity of normal mice treated with fluorescent rabbit anti-mouse immunoglobulin. x690.

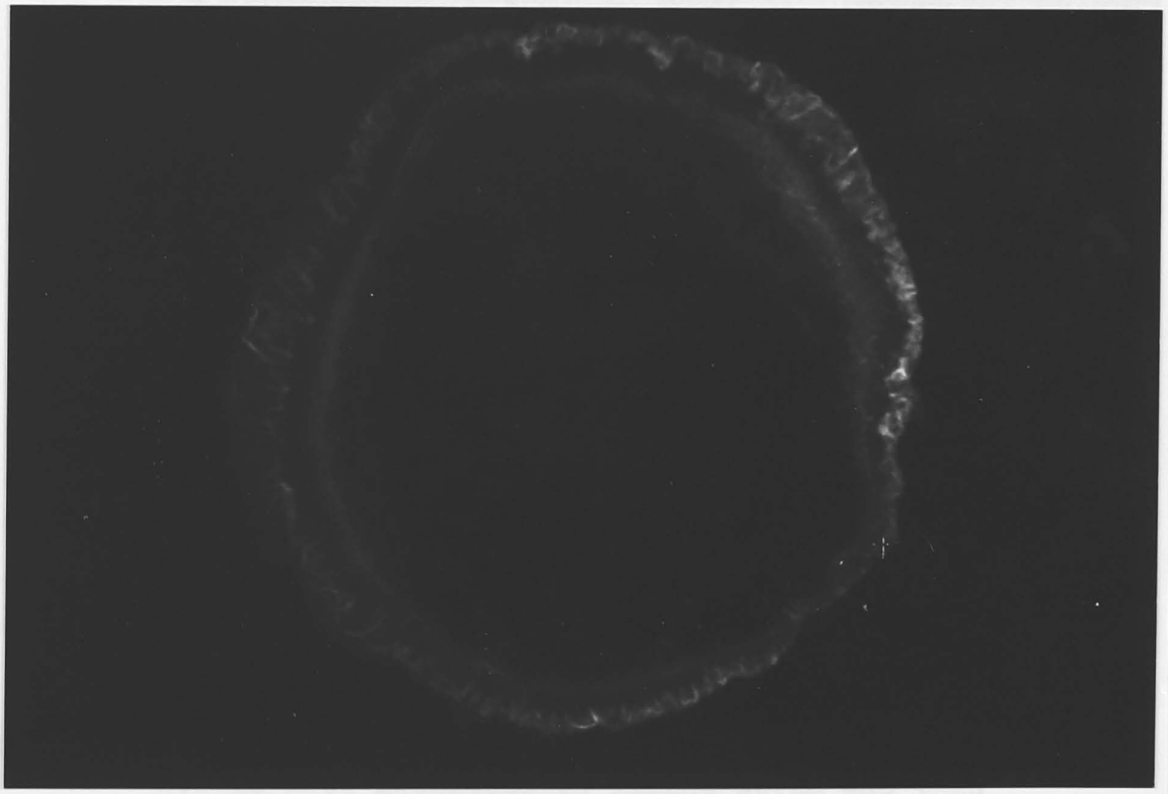


Plate 23 A tetrathyridium from the liver of normal mice treated with fluorescent rabbit anti-mouse immunoglobulin. x1000.

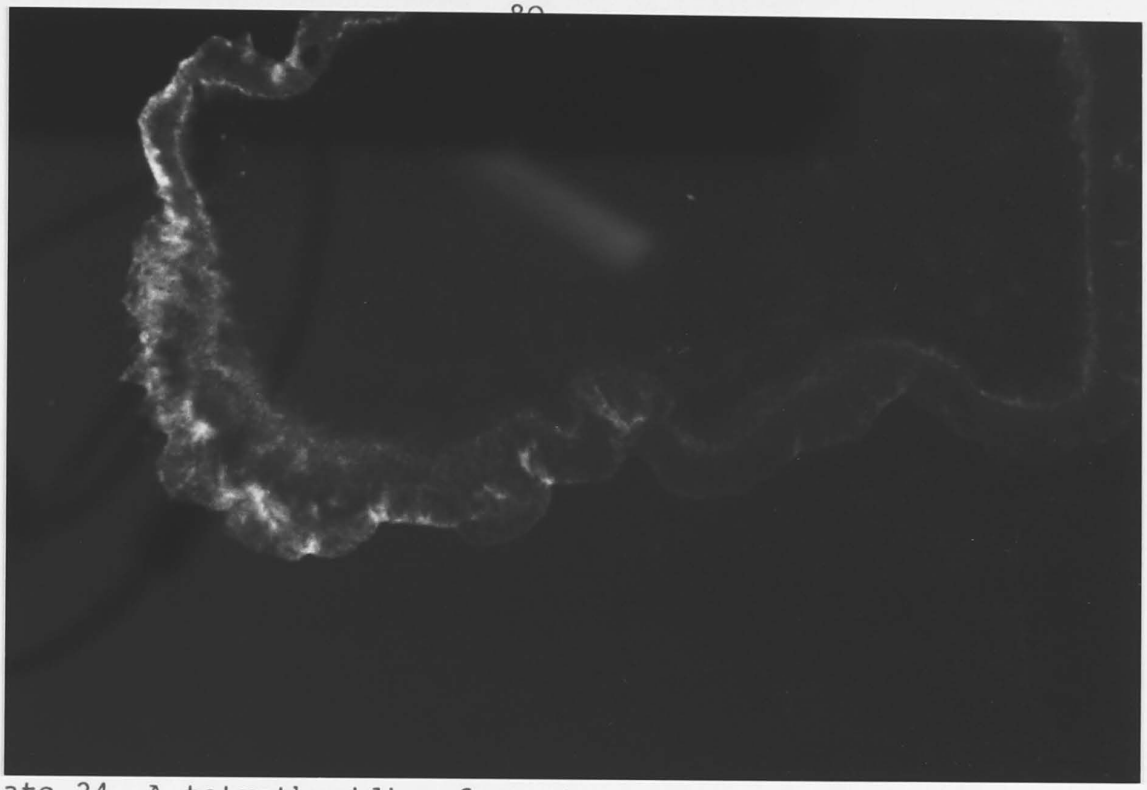


Plate 24 A tetrathyridium from the peritoneal cavity of athymic mice treated with fluorescent rabbit anti-mouse immunoglobulin. x690.

Plate 25 A tetrathyridium from the peritoneal cavity of normal mice incubated in immune serum and then treated with fluorescent rabbit anti-mouse immunoglobulin. x690.

The plaque forming cell technique proved to be quite successful in testing for antibody form cells in mice against tetrathrydial antigens. Strong positive results were obtained using spleen cells assayed against the ES antigens of the parasite. The plaques detected have been shown (by the inhibition test) to be specifically caused by the ES antigens of the parasite. As expected, the total number of antibody forming cells greatly increases in the infected mouse, due to a non specific stimulation by the parasite of antibody production. The antibodies produced during the first 4 weeks of infection are mainly of the IgM type, as indicated from the indirect IgG plaque forming cell assay. On using the developer, the number of plaques is reduced to 50% of the number obtained without the developer. Since the developer only forms large inhibitory complexes with 50% of the IgM molecules, the remaining active IgM antibodies accounts for the plaques formed. Hence, summing up all the results from these assays, the plaque forming cell technique shows that during the first 4 weeks of infection with M. corti tetrathyridia, the mouse produces antibodies of the IgM type against the excretory and secretory products of the parasite, mainly in the spleen.

With the Indirect haemagglutination and plaque forming cell tests, glutaraldehyde and $CrCl_3$, respectively, were used to attach antigen molecules on to SRBC. When using such antigen fixing agents only certain molecules of the parasite antigens will conjugate to the SRBC. This may cause a lack of sensitivity in these two tests.

EXPERIMENT 5

THE EFFECT IN MICE OF PASSIVE IMMUNISATION WITH SERUM ON TETRATHYRIDIAL MULTIPLICATION

The injection of immune serum from mice infected for 12 weeks with M. corti transfers immunity to recipient mice, infected with tetrathyridia at the time of serum transfer (Kowalski and Thorson, 1972a). This passive immunity stems from serum antibodies raised in the donor mice against tetrathyridial antigens. Kowalski and Thorson's experiments leave open the question of whether the protective action of antibodies requires the help of thymus cells influenced lymphocytes (T-cells). To study whether T-cells are required for passive immunization in mice against M. corti, serum was transferred to mice genetically lacking a thymus (athymic mice) and the rate of tetrathyridial multiplication compared in these mice in the presence and absence of immune serum.

Ten normal and 10 athymic female BALB/c mice (age 8 weeks) were each infected with 20 tetrathyridia. Five mice from each group received intraperitoneally 0.5 ml normal serum obtained from normal Piebald mice. The other 5 mice in each group received intraperitoneally 0.5 ml immune serum, obtained from 12 week infected Piebald mice. (Piebald mice were used as a large stock of this strain of mice both non infected and infected with M. corti were available at the Zoology Department). All the recipient mice were killed 10 days post infection and serum transfer, and the tetrathyridia recovered from each mouse were counted (see Table 16).

The effect of serum transfer at the time of infection with M. corti in both normal and athymic mice was also studied at day 20 post infection. Fifteen normal and 15 athymic female BALB/c mice were each infected with 20 tetrathyridia. Five mice from each group received intraperitoneally 0.5 ml normal serum from uninfected CBA/H mice, another 5 mice from each group received 0.5 ml immune serum from 12 week infected CBA/H mice with tetrathyridia and the remaining 5 mice in each group received no serum transfer. All the mice were killed 20 days

TABLE 16 Attempted passive immunization with serum of normal and athymic BALB/c female mice infected with 20 tetrathyridia

Type of mouse (5 mice per group)	Infection Period (days)	Serum (0.5 ml i.p*)	Mean number of tetrathyridia (+ range)	Multiplication factor of tetrathyridia
Exp 1				
Normal	10	Normal	117 (95-145)	5.9
Normal	10	Immune	65 (47-84)	3.3
Athymic	10	Normal	123 (48-192)	6.2
Athymic	10	Immune	40 (6-56)	2.0
Exp 2				
Normal	20	None	126 (75-213)	6.3
Normal	20	Normal	220 (121-331)	11.0
Normal	20	Immune	142 (18-266)	7.1
Athymic	20	None	1309 (918-1595)	65.5
Athymic	20	Normal	1148 (1020-1263)	57.4
Athymic	20	Immune	967 (249-1529)	48.3

Both normal and athymic mice can be passively immunized with immune serum as shown in the results obtained from mice killed 10 days post treatment.

* intraperitoneally

post treatment and the tetrathyridia recovered from each mouse were counted (see Table 16).

Tetrathyridia incubated in vitro in immune serum become coated with a precipitate (Kowalski and Thorson, 1972a) showing that antibodies can act directly on to the parasite. To see whether these attached antibodies can effect the rate of tetrathyridial multiplication, antibody coated tetrathyridia were injected into recipient mice.

Tetrathyridia were incubated for 24 hours at 37°C in groups of 20 in either 1 ml normal serum (from uninfected CBA/H mice) or immune serum (from 12 week infected CBA/H mice with tetrathyridia) or saline. Three groups of 5 mice each (male, CBA/H, age 8 weeks) were all infected with 20 tetrathyridia incubated in either normal serum or immune serum or saline respectively. The tetrathyridia were very gently washed with saline from the incubating sera before injected into the recipient mice. The tetrathyridia which had been incubated in the immune serum were coated with a precipitate, visible under the light microscope, most evident around the scolex area. All the mice were killed 10 days post infection and the tetrathyridia recovered from each mouse were counted (see Table 17).

The immune serum used for experiments on the passive transfer of immunity was obtained from 12 week infected mice. Antibody formation has been detected at an earlier date (see experiment 4). To find out how soon serum contains sufficient antibody to give protection an experiment was undertaken in which sera from mice infected for different periods of time was used for passive immunization.

Female BALB/c mice (age 8 weeks) were each infected with 20 tetrathyridia. Six groups of 10 mice were killed on weeks 0, 2, 6, 9, 10 and 12 post infection to supply serum (obtained as described in Methods P.9). Six groups of female BALB/c mice (5 mice per group, age 8 weeks) were each infected with 20 tetrathyridia and received intraperitoneally 0.5 ml serum from one of the above donor groups of mice. All mice were killed 10 days post treatment, and tetrathyridia recovered from each mouse were counted (see Table 18).

TABLE 17 Effect of an antibody layer adsorbed in vitro prior to tetrathyridial inoculation on their subsequent rate of multiplication in the mouse

Incubating medium (1 ml containing 20 tetrathyridia for 24 hours)	Number of mice (CBA/H, male, age 8 weeks)	Presence of adsorbed antibodies around tetrathyridia	Mean number of tetrathyridia recovered 10 days post infection (+ range)
Saline	5	-	73 (10-97)
Normal serum	5	-	83 (27-136)
Immune serum	5	+	45 (14-86)

An ANOVA showed that the means are not significantly different, (see Appendix P.III)

TABLE 18 Transfer of serum obtained from mice infected for different periods of time to recipient mice infected with 20 tetrathyridia at time of serum injection

Serum from donor mice infected for: (weeks)	Number of BALB/c recipient mice (female, age 8 weeks)	Mean number of tetrathyridia recovered 10 days post infection (+ range)
0	5	118 (56-200)
2	5	93 (55-110)
6	5	72 (52-136)
9	5	71 (12-114)
10	5	61 (18-114)
12	5	48 (24-65)

It was shown statistically, using a one tailed T-test, that only the serum from 12 week infected donors can passively immunize infected recipient mice (see Appendix P.112)

Conclusions

Immune serum passively immunizes both normal and athymic mice and it can therefore be concluded that antibodies can limit tetrathyridial multiplication without the help of T-cells. However the effect of the immune serum was only clearly detected, within the first 10 days of infection.

When antibody coated tetrathyridia are injected into mice, the tetrathyridia multiply at a normal rate showing that the adsorbed antibodies have no measurable effect on tetrathyridial multiplication. Such antibody may not be persistent in the host so that a negative result does not necessarily mean that such antibody is ineffective. It may simply mean that the effect is transient.

Passive immunization is only statistically demonstrated with serum from donor mice infected for 12 weeks. However there is a gradual decrease in tetrathyridial recovery with serum used from donor mice infected with increased periods of time.

EXPERIMENT 6

IMMUNIZATION OF MICE TO TETRATHYRIDIA OF M. CORTI

Previous experiments have shown that the tetrathyridia of M. corti multiply at a much faster rate in athymic mice than in normal mice (Pollacco, 1975). This indicates that an immunological response occurs in normal mice which slows down the rate of multiplication of the parasite, but which is unable to prevent the eventual death of the mouse from the massive lesions occurring in the liver and elsewhere, due to the heavy parasite burden. Attempts by previous workers have been made (Kowalski and Thorson, 1972b; Kazacos, 1976) to immunize mice. They were successful in limiting tetrathyridial multiplication but complete immunization of the host was not achieved. Hence experiments were undertaken to see if this natural immunity can be stimulated to the point where complete protection is achieved. Mice were immunized as follows:-

A. Immunization of mice using irradiated parasite

Preliminary experiments (Pollacco, 1975) have shown that irradiation of the parasite with 80 k Rads using a ^{60}Co source (as explained in Methods P.24) prevents the parasite from multiplying. In experiment (i), 14 male Quackenbusch mice were each infected with 100 irradiated tetrathyridia (80 k Rads). Twelve weeks later, 7 mice were challenged with 100 normal tetrathyridia. All mice were killed 10 days later and the tetrathyridia recovered were counted (see Table19).

In experiment (ii) 5 male Quackenbusch mice were infected with 20 irradiated tetrathyridia. Twelve weeks later, these mice together with 5 male Quackenbusch mice were infected with 20 normal tetrathyridia. The mice were killed 10 days later and the tetrathyridia recovered were counted (see Table19).

B. Vaccination with the bacillus Bacille Calmette-Guerin (BCG)

Vaccination with Mycobacterium bovis strain BCG has been found to suppress tumour growth in both animals and man (Wolf, Tracey, and Henney, 1976). It has been reported that

TABLE 19 The immunization of mice by γ-irradiated tetrathyridia. Intraperitoneal injection of irradiated tetrathyridia was followed 12 weeks later by the injection of normal tetrathyridia

		Number of Quackenbusch mice (male, age 8 weeks)	Number of irradiated parasites (80 k Rads)	Number of normal parasites used in challenge 12 weeks later	Parasites recovered 10 days after challenge (Mean ± S.E.)
Experiment (i)	a)	7	100	-	3 ± 2
	b)	7	100	100	110 ± 26
Experiment (ii)	c)	5	20	20	63 ± 14
	d)	5	-	20	161 ± 20

Using Student's T test, group (c) is significantly different from group (d) at $P < 0.01$ (see Appendix P.114)

TABLE 20 Immunization of mice with Bacille Calmette-Guerin (BCG) followed by an infection with tetrathyridia one week later

Number of ♀,H mice	Administration of BCG i.p.*	Administration of saline i.p.*	Number of tetrathyridia	Tetrathyridia recovered 16 days post infection (Mean ± S.E.)
6	None	1 ml	30	327 ± 99
6	Given $20x10^6$	None	30	274 ± 34

* intraperitoneally

Using Student's T test, the 2 groups are not significantly different at the 5% level of significance (see Appendix P. 114)

BCG may affect tumours through stimulation of the host's macrophages. However, Wolfe et al, (1976) also speculate that BCG may act via a non-macrophage population of cytotoxic cells. Clark, Allison and Cox (1976) completely protected mice against Babesia microti and Babesia rodhani by a previous infection with BCG. They suggest that BCG protects mice by increasing the release of non-antibody soluble mediators of immunity. To see whether mice can be similarly immunized with BCG against tetrathyridial infections, the following experiment was set up:-

Six female H mice were given 1 ml BCG (CSL) containing $20x10^6$ bacteria, intraperitoneally. Six control female H mice received 1 ml saline intraperitoneally. A week later all the mice were infected with 30 tetrathyridia. On days 5, 9 and 13 after infection with tetrathyridia, the mice were injected subcutaneously with trypan blue (2.5 mg per 20 gm mouse weight - trypan blue prepared as 0.5% suspension in saline). This dye was used to show up macrophages, which take up the dye, in the histological sections which were prepared as explained below. The mice were killed 16 days post infection and the tetrathyridia recovered from both the liver and the peritoneal cavity were counted (see Table 20).

Small pieces from the livers of 3 mice from each group were taken for histology. These were fixed in Bouin's, blocked, sectioned and stained with Hematoxylin and Eosin, and Carmalum stains (see Methods P. 27) to compare the numbers of macrophages in the BCG treated mice with those of the controls.

Conclusions

Mice can be partially immunized against tetrathyridial multiplication by a primary infection with irradiated parasites which are unable to multiply but are still metabolically active.

Treating mice with BCG does not effect tetrathyridial multiplication. No apparent increases in the macrophage population were revealed in the stained liver sections of mice receiving either BCG or saline treatment prior to infection.

DISCUSSION

Mice infected with the intermediate stage of the cestode M. corti (the tetrathyridium) develop an immunity which is both B-cell and T-cell dependent. Normal mice, as described by Kowalski and Thorson (1972a), were partially immunized against tetrathyridia by the transfer of serum obtained from mice parasitised for 3 months with M. corti. A similar protection was obtained in athymic mice suggesting that the protective effects of antibody are not T-cell dependent. The passive immunization of mice by serum immediately before infection is clearly demonstrated 10 days after infection but the effects are not clearly demonstrable 20 days post treatment.

The question of whether the antibody response to M. corti is T-cell dependent was investigated using athymic mice. However the results were inconclusive because precipitating and reagenic antibodies were not demonstrable until the 3rd and 12th week of infection respectively, which exceed the life span of the infected athymic mouse. Agglutinating antibodies are possibly present in the athymic mouse, but if so these were at too low a level to be detected with certainty. From experiments with fluorescent antibody, it would appear that the infected athymic mice, unlike normal mice, do not produce immunoglobulins which coat the tegument of the parasite when present in the peritoneal cavity. These immunoglobulins may be the same as the immunoglobulins (mainly IgG) reported by Mitchell, Marchalonis, Smith, Nicholas and Warner (1972) bound on the surface of tetrathyridia in the peritoneal cavity of normal mice. Since these immunoglobulins are not present on the surface of tetrathyridia from the peritoneal cavity of athymic mice, it may be possible that the mouse may require help from T-cells to produce at least some antibodies against the antigens of M. corti.

M. corti multiplies asexually very rapidly in athymic mice (50 to 70 times) during the first 20 days of infection

when compared with its multiplication rate in normal mice (6 to 8 times) of the same inbred strain. The histology of the liver provides an explanation for the susceptibility of the athymic mouse. Specht and Widmer (1972) have observed the liver pathology in the normal mouse. Between the 10th and 15th day after infection normal mice develop an extensive inflammatory reaction to the tetrathyridia andlay down collagen fibres in the liver. Only a slight inflammatory reaction develops in the athymic mouse, but the fibrogenesis and the extensive inflammatory reaction can be restored by the transfer of either spleen or thymus cells suspension. The thymus cells were obtained from unparasitised mice, and the spleen cells were effective whether obtained from parasitised or unparasitised mice. Athymic mice can produce collagen in the liver to lesions caused by a heated cautery knife. These results indicate that the formation of collagen and an extensive inflammatory reaction in the liver of infected mice is a T-cell dependent reaction.

To study the role played by different cell populations, mice were irradiated at 750 rads to inactivate the immune system and then reconstituted with cells by grafts from various organs from normal mice. The transfer of either bone-marrow cells or peritoneal B-cells to irradiated mice lowered the rate of tetrathyridial multiplication, while the transfer of thymus cells or peritoneal T-cells stimulated tetrathyridial proliferation. This seems to contradict the previous results where T-cells were important in limiting tetrathyridial proliferation by the stimulation of an extensive inflammatory reaction and collagen formation in the liver. However, the stimulatory effect by T-cells in irradiated mice was observed within the first 10 days of infection, when collagen formation had not been observed. Unfortunately the numbers of irradiated mice used in these cell transfer experiments were small because of the limited supply of suitable mice. Moreover, no statistically significant difference was observed in the rate of tetrathyridial multiplication in athymic mice and normal mice of the same inbred strain

during the first 10 days of infection (Pollacco, 1975) so that the role of T- and B-cells during this infection period cannot be concluded with certainty.

Tetrathyridial multiplication was suppressed in irradiated or normal mice receiving peritoneal cell transfers from unparasitised or parasitised donor mice respectively. Consequently peritoneal cell populations were investigated by staining peritoneal cells smears. By the third week of infection, a drastic increase in the numbers of macrophages, eosinophils, and neutrophils was seen, while the lymphocyte count decreased. The increase in the phagocytic cells may be responsible for the sequestration and local destruction of M. corti antigens, which occurs in the peritoneal cavity of mice (Mitchell and Handman, in press). The sequestration is highly T-cell dependent (Mitchell, G.F., unpublished) and therefore it would be of importance to study the peritoneal cell population of the athymic mouse 3 weeks after infection.

Using the fluorescent antibody technique on histological sections of tetrathyridia, it can be demonstrated that serum from 12 week infected normal mice contains antibodies directed against the parasite, probably the excretory and secretory structure. This is consistent with the results of Kowalski and Thorson (1972b) and Kazacos (1976) which indicated that it is probably the excretory and secretory antigens of the parasite that is the protective antigen. My experiments with the plaque cell technique showed that very few plagues developed against M. corti somatic antigen while quite a number developed against the excretory and secretory antigen of the parasite. This also suggests that the host may not react strongly or in a protective manner to the somatic antigen of the parasite.

Specht and Widmer (1972) observed the infiltration of the peripheral layers of the collagenous capsule encapsulating the tetrathyridia in the liver by eosinophils and plasma cells about the 8th week of infection. This was

accompanied by lobular parenchymal regeneration of the liver tissue, and they suggest that late in the infection, the host acquires a resistance to the parasite which was not evident earlier in the infection. This resistance limits continued parenchymal destruction by physical restriction of the parasite. My serological tests show that the time when the host aquires resistance coincides with the appearance of IgE antibodies, and the development of an immune serum which when transferred to recipient mice can partially protect them against tetrathyridial infections. Passive immunization, previously demonstrated by Kowalski and Thorson (1972a) may therefore involve reagenic antibodies. However IgE antibody response is highly dependent on antigen-specific T-cells and collaboration between T- and B-cells is required for the formation of IgE antibodies (Ishizaka, 1976).

It is of interest to see that in several hosts the immediate-type reactivity to helminth antigens is mediated by IgE antibodies (Catty, 1969). These antibodies are produced in rats infected with N. brasiliensis (Ogilvie, 1964; Bloch and Wilson, 1968) and S. mansoni (Ogilvie, 1964; Ogilvie et al., 1966), in rabbits infected with Ascaris (Hogarth-Scott, 1967). Although the demonstration of reagins in helminth infected hosts may be a coincidental expression of infection, it may be true, as has frequently been suggested, that reagin production is closely associated also with the duration and site of antigenic contact with the host. In the case of gastro-intestinal infestations, local reagin production may be a key factor in resistance. It has been suggested by Kowalski and Thorson (1972a) that orally infected mice with M. corti do exhibit a greater resistance to the parasite than mice infected by an intraperitoneal injection of tetrathyridia. However, it is not yet known whether this increase in resistance coincides with an increase in the IgE antibody production in the mouse similar to that seen in several other helminths.

In summary, the mouse's immunological response to M. corti can be divided into at least two stages and probably more:

a) A collagenous inflammatory reaction, occurring in the liver, which begins after the 10th day of infection. Both the collagen formation and the inflammatory reaction limit the parasites' movement and multiplication, because of the severe alterations in the liver substrate (Specht and Widmer, 1972). This reaction is T-cell dependent.

b) A reaction involving antibody production effective after the 8th week of infection which restricts parasites movement and limits liver destruction (Spect and Widmer, 1972). This reaction probably involves reagenic antibodies and is T-cell dependent for their production.

APPENDIX

STATISTICAL ANALYSIS OF DATA

Experiment 2 Cellular transplants to intact, irradiated and athymic mice

Ai. Transfer of peritoneal cells to normal mice

Groups	N	$\bar{x}$	S^2
a) Receiving no cell transfer	5	130	2347
b) Receiving "immune" peritoneal cells	5	73	678
c) Receiving normal peritoneal cells	5	145	4993

A Bartlett's Chi-Sq. test (program written by Dr. Marples) was carried out to test for variance distribution.

Chi-Sq.	df.	Probability
3.7	2	$P > 0.05$

Since the variances are not significantly different, a Student's T test (for equal variances, program written by Dr. Marples) was carried out.

Comparison	df.	T	Significance
Group (a) receiving no cells with group (c) receiving normal peritoneal cells	8	0.38	$P > 0.05$
Group (a) with group (b) receiving "immune" peritoneal cells	9	2.35	$P < 0.05$
Group (c) with group (b)	9	2.55	$P < 0.05$

The mean for mice receiving "immune" peritoneal cells is significantly different from the other 2 means at the 5% level of significance.

Aii Transfer of tetrathyridia together with adherent peritoneal cells to normal mice

Groups	N	$\bar{x}$	S^2
Washed tetrathyridia	5	246	1583
Tetrathyridia covered with peritoneal cells	5	178	3426

An F test for equality of variances (Snedecor, 1967) was carried out and these were found to be equally distributed ($P < 5\%$). A Student's T-test was carried out to compare the means of the groups receiving tetrathyridia with and without adherent peritoneal cells.

df.	T	Significance
8	2.1	$P > 0.05$

The difference between the means is not significantly different at the $P = 0.05$ level.

EXPERIMENT 2B Transfer of cells to irradiated mice

2Bi)

Groups	Code	N	$\bar{x}$
Normal	N	6	61.5
Irradiated	R	6	56
Irradiated + thymus cells	RT	7	66
Irradiated + bone marrow cells	RB	5	78.8
Irradiated + bone marrow and thymus cells	RBT	7	61.6
Irradiated + spleen cells	RS	6	36
Irradiated + lymph node cells	RL	5	30.6
Irradiated + peritoneal cells	RP	4	21

Analysis of variance (program written by Dr. Marples)

ANOVA	SS	df	MS	Fraction	P
Among groups	13931	7	1990	5.168	0.0003
Within	14632	38	385		

Least significant difference, $LSD = Q_{\alpha}\,(k.df)\sqrt{MS_{within}}\sqrt{\frac{n_1+n_2}{2n_1.n_2}}$

$= Q_{\alpha}\,(k.38)\quad 19.62\sqrt{n_1 + n_2/2n_1n_2}$

EXPERIMENT 2Bi (cont.)

$Q_{0.05}$ values for difference between ranks

	k =	2	3	4	5	6	7	8
$Q_{0.05}$ (k 40)	=	2.858	3.442	3.791	4.039	4.237	4.521	4.5
$Q_{\alpha}\sqrt{MS}$	=	56.1	67.5	74.3	79.2	83.1	86.1	88.
LSD								
n5, n4 = 0.474		26	32	35	37	39	41	42
n5, n6 = 0.428				32	34			
n4, n7 = 0.443						37		
n4, n6 = 0.456				34	36			
n7, n5 = 0.414					34	34		
n7, n6 = 0.393				29	31			

EXPERIMENT 2Bi (cont.)

$Q_{0.01}$ values for difference between ranks from table of critical values of studentised range (Sokal and Rohlf, 1969).

k (Rank dif + 1) =	2	3	4	5	6	7	8
$Q_{0.01}(k, 40)$ =	3.825	4.367	4.696	4.931	5.114	5.265	5.39
$Q_{\alpha}\sqrt{MS}$	75	85.68	92.14	96.75	100.75	103.3	105.9
LSD n5, n4 / $\sqrt{9/40}$ = 0.474							50
LSD n5, n5 / $\sqrt{10/50}$ = 0.447						46	
LSD n5, n6 / $\sqrt{11/60}$ = 0.428		37	39	41	43		
LSD n7, n4 / $\sqrt{11/56}$ = 0.443					42	45	
LSD n7, n5 / $\sqrt{12/70}$ = 0.414				40	42	42	
LSD n6, n4 / $\sqrt{10/48}$ = 0.456				44			
LSD n7, n6 / $\sqrt{13/84}$ = 0.393				38			
LSD n6, n6 / $\sqrt{12/72}$ = 0.408			38				
LSD n7, n7 / $\sqrt{14/98}$ = 0.378	28	33	34	31	38	40	40

EXPERIMENT 2Bi (cont.)

Table of difference between means

Rank	Code	n	$\bar{x}$	Rank	1	2	3	4	5	6	7	8
				Code	RP	RL	RS	R	N	RBT	RT	RB
				$\bar{x}$	21	31	36	56	62	62	66	79
				n	4	5	6	6	6	7	7	5
1	RP	4	21									
2	RL	5	31		10	-						
3	RS	6	36		15	5	-					
4	R	6	56		35*	25	20	-				
5	N	6	62		41*	31	26	6	-			
6	RBT	7	62		41*	31	26	6	-	-		
7	RT	7	66		45**	35*	30	10	4	4	-	
8	RB	5	79		58**	48**	43**	23	17	17	13	

* = sig. P = 0.05 ** = sig. P = 0.01

Irradiated mice receiving peritoneal cells develope significantly few tetrathyridia than normal mice, irradiated mice given B-cells, T-cells, both T- and B-cells or untreated irradiated mice. There are large differences in the means between irradiated mice receiving spleen or lymph node cells and irradiated unsupplemented or normal mice, but do not quite reach P = 0.05 level of significance.

EXPERIMENT 2Bii

Groups	Code	N	$\bar{x}$
Normal	N	4	66.8
Irradiated	R	5	54.6
Irradiated + thymus cells	RT	6	119.5
Irradiated + bone marrow cells	RB	6	24.2
Irradiated + thymus and bone marrow cells	RBT	6	143
Irradiated + peritoneal cells	RP	5	37.6
Irradiated + peritoneal cells depleated in macrophages	RP-M	5	25.8

ANOVA	SS	df	MS	Fraction	P
Among groups	75520	6	12587	19.496	less than
Within groups	19368	30	645.6		0.001

Least significant difference:

$$LSD = Q_{\alpha}\ (k.df)\ \sqrt{MS_{within}}\sqrt{\frac{n_1 + n_2}{2n_1 \cdot n_2}}$$

$$= Q_{\alpha}\ (k.30)\ 25.41\sqrt{n_1 + n_2/2n_1\ n_2}$$

EXPERIMENT 2Bii (cont.)

$Q_{0.05}$ for difference between Ranks from table of critical values of Studentised Range. k = (difference in ranks + 1).

k =	2	3	4	5	6	7
$Q_{0.05}$ (k . 30) =	2.888	3.485	3.845	4.102	4.302	4.464
$Q_{\alpha}\sqrt{MS}$ =	73.4	88.6	97.7	104.2	109.3	113.4
LSD						
n6, n6 = 0.408	30				45	46
n6, n5 = 0.428		38	42	45	47	
n5, n5 = 0.447	33	40	44	47	49	51
n5, n4 = 0.474		40	46			
n6, n4 = 0.456	33	40		48		
$Q_{0.01}$ (K. 30) =	3.889	4.455	4.799	5.048	5.242	5.401
$Q_{\alpha}\sqrt{MS}$ =	98.8	113.2	121.9	128.2	133.2	137.2
LSD						
n6, n6 = 0.408					54	60
n6, n5 = 0.428		48	52	55	57	
n6, n4 = 0.456	45	52				

EXPERIMENT 2Bii (cont.)

Table of difference between means

Rank	1	2	3	4	5	6	7
Code	RB	RP-M	RP	R	N	RT	RBT
n	6	5	5	5	4	6	6
$\bar{x}$	24.2	25.8	37.6	54.6	66.8	119.5	143

Rank	Code	n	$\bar{x}$						
1	RB	6	24.2	-					
2	RP-M	5	25.8	1.6	-				
3	RP	5	37.6	13	12	-			
4	R	5	54.6	30	29	17	-		
5	N	4	66.8	43	41	29	12	-	
6	RT	6	119.5	95**	94**	82**	65**	53**	-
7	RBT	6	143	119**	117**	105**	88**	76**	24	-

** Sig. at P = 0.001, other differences not significant at P = 0.05 or P = 0.01

The irradiated mice receiving thymus cells or thymus cells plus bone marrow cells have means highly significantly different from the remaining mice. The irradiated mice receiving peritoneal cells though lower than untreated irradiated mice are not significantly so. The small number of normal mice makes the comparisons with this group of little significance.

EXPERIMENT 2Biii

Groups	Code	N	$\bar{x}$
Normal	N	5	106.8
Irradiated	R	4	114
Irradiated + peritoneal cells	RP	4	72
Irradiated + peritoneal cells depleted in macrophages	RP-M	4	80
Irradiated + peritoneal T-cells	RPT	4	121.5
Irradiated + peritoneal B-cells	RPB	4	66.25
Irradiated + peritoneal T- and B-cells	RPTB	5	59.2

ANOVA	SS	df	MS	Fraction	P
Among groups	18146	6	3024	6.473	less than
Within groups	10746	23	467		0.001

Least significant difference:

$$LSD = Q_{\alpha}\ (k.df) \sqrt{MS_{within}} \sqrt{\frac{n_1 + n_2}{2n_1 \cdot n_2}}$$

$$= Q_{\alpha}\ (k.23)\ 21.61 \sqrt{n_1 + n_2 / 2n_1\ n_2}$$

EXPERIMENT 2Biii (cont.)

$Q_{0.01}$ values for difference between ranks from table of critical values of studentised range

k (Rank diff. + 1)	2	3	4	5	6	7
$Q_{0.01}$ (k.24)	3.956	4.546	4.907	5.168	5.374	5.542
$Q_{\alpha}\sqrt{MS}$	85	98.2	106	111.7	116.1	119.7
LSD						
n5 n4 = 0.474				50	55	56
n4 n4 = 0.5				56	58	

$Q_{0.05}$ values for differences between ranks

k =	2	3	4	5	6	7
$Q_{0.05}$ (k.24)	2.919	3.532	3.901	4.166	4.373	4.541
$Q_{\alpha}\sqrt{MS}$	63.1	76.3	84.3	90	94.5	98.1
LSD						
n5 n4 = 0.474			40		45	47
n4 n4 = 0.5		38	42	45	47	
n5 n5 = 0.447				40		

EXPERIMENT 2Biii (cont.)

Rank	Code	n	$\bar{x}$
1	RPTB	5	59.2
2	RPB	4	66.25
3	RP	4	72
4	RP-M	4	80
5	N	5	106.8
6	R	4	114
7	RPT	4	121.5

Table of difference between means

Rank	1	2	3	4	5	6	7
Code	RPTB	RPB	RP	RP-M	N	R	RPT
n	5	4	4	4	5	4	4
$\bar{x}$	59.2	66.25	72	80	106.8	114	121.5
1	-						
2	7	-					
3	13	6	-				
4	21	14	8	-			
5	48*	41*	35	27	-		
6	55**	48*	42*	34	7	-	
7	82**	55*	50*	42*	15	8	-

* Significantly different at P = 0.05

** Significantly different at P = 0.01

Normal mice have a mean significantly different from irradiated mice receiving peritoneal T- and B-cells or peritoneal B-cells. Irradiated mice receiving peritoneal cells or peritoneal B-cells have means which differ significantly from irradiated mice and irradiated mice receiving peritoneal T-cells.

EXPERIMENT 2c Transfer of spleen cells to athymic mice

Groups	Code	N	Peritoneal Cavity		Liver		Total	
Athymic mice receiving spleen cells from:-			$\bar{x}$	S^2	$\bar{x}$	S^2	$\bar{x}$	S^2
a) 5 week infected donor mice	A5WPST	6	160	13017.07	355	39140	515	79806.2
b) 3 week infected donor mice	A3WPST	6	181	62601.1	291	40832.7	494	186105.1
c) 1 week infected donor mice	A1WPST	6	217	14986.4	419	67574.7	635	74092.8
d) Unparasitised donor mice	AnST	6	304	61906.9	404	45797.5	708	202320
e) No spleen cells	A	6	668	245092.7	4351	613271	5018	8301889.7
f) Normal mice receiving no spleen cells	N	6	323	29379.5	364	7131.8	687	49239.6

A Bartlett's Chi-Sq test was carried out to test for variance homogeneity.

	Chi-Sq	df.	Probability
Peritoneal cavity	14.72	5	< 0.025
Liver	72.6	5	< 0.001
Total	54.12	5	< 0.001

The variances are significantly different at P = 0.05. However when group A is excluded, the variances of the remaining groups are not significantly different.

	Chi-Sq	df.	Probability
Peritoneal cavity	4.95	4	> 0.25
Liver	5.05	4	> 0.25
Total	3.52	4	> 0.25

Because of unequal variances, the recoveries were transformed to their natural logarithms (presupposes that the variance is a function of the mean).

Group	N	Peritoneal cavity		Liver		Total	
		Mean Log_n ± SE	S^2	Mean Log_n ± SE	S^2	Mean Log_n ± SE	S^2
A5WPST	6	4.82±0.340	0.69	5.716±0.264	0.65	6.091±0.259	0.41
A3WPST	6	4.72±0.475	1.61	5.41 ±0.354	0.76	5.845±0.404	0.98
AlWPST	6	5.20±0.291	0.5	5.83 ±0.308	0.58	6.37 ±0.185	0.2
AnST	6	5.18±0.576	1.99	5.75 ±0.353	0.74	6.23 ±0.423	1.06
A	6	6.04±0.562	1.38	8.05 ±0.464	1.14	8.183±0.474	1.16
N	6	5.56±0.359	0.77	5.88 ±0.0929	0.05	5.49 ±0.0971	0.1

A Bartlett's Chi-Sq test was carried out.

	Chi-Sq	df.	Probability
Peritoneal cavity	3.73	5	> 0.5
Liver	9.3	5	> 0.1
Total	9.97	5	> 0.05

Bartlett's test for logarithmically transformed recoveries shows variances are not significantly unequal.

An ANOVA was carried out.

	SS	df.	MS	Fraction	P
Peritoneal cavity					
Among group	7.317	5	1.4633	1.217	0.325
Within group	36.078	30	1.2168		
Liver					
Among group	28.061	5	5.612	8.792	3.18×10^{-5}
Within group	19.149	30	0.6383		
Total					
Among group	21.036	5	4.207	6.226	4.51×10^{-4}
Within group	20.271	30	0.6757		

The peritoneal populations of tetrathyridia from all the groups do not differ significantly.

The Mann-Whitney non-parametric comparison between pairs of means have shown that groups:

A and A5WPST are significantly different	$P < 0.05$
A and AnST are significantly different	$P < 0.05$
A and N are significantly different	$P < 0.05$
A5WPST and AnST are not significantly different	$P > 0.05$
A5WPST and N are not significantly different	$P > 0.05$

EXPERIMENT 5 The effects in mice of passive immunization with serum on tetrathyridial multiplication

A. Effect of an antibody layer adsorbed in vitro prior to tetrathyridial inoculation on their subsequent rate of multiplication in the mouse

Groups	N	$\bar{x}$	S^2
Mice infected with:			
a) Tetrathyridia incubated in saline	5	73	1317.2
b) Tetrathyridia incubated in normal serum	5	83	1574.7
c) Tetrathyridia incubated in immune serum	5	45	1107.8

To test for homogeneity of variance Bartlett's Chi-Sq test was carried out.

Chi-Sq	df.	Probability
0.11	2	> 0.9

As the variances are not significantly different at P = 0.05, an ANOVA was done.

	DoF	SS	MS	Fraction	P
Among group	2	3924.1	1962.1	1.47	0.268
Within group	12	15998.8	1333.2		

The differences of none of the groups are significantly different at P = 0.05

B. Transfer of serum obtained from mice infected for different periods of time to recipient mice infected with 20 tetrathyridia at time of serum injection

Groups	N	Peritoneal Cavity		Liver		Total	
		$\bar{x}$	S^2	$\bar{x}$	S^2	$\bar{x}$	S^2
a) Mice receiving serum from uninfected donors	5	22	218.80	96	2504.8	118	3928
b) Mice receiving serum from 2 week infected donors	5	14	36.30	58	720.70	72	680.30
c) Mice receiving serum from 6 week infected donors	6	14	133.47	58	1119.07	72	1409.6
d) Mice receiving serum from 9 week infected donors	6	6	161.60	58	1361.07	71	1842.67
e) Mice receiving serum from 10 week infected donors	6	9	175.6	52	674.57	61	1322.57
f) Mice receiving serum from 12 week infected donors	5	9	38	39	397.7	48	645.7

A Bartlett's Chi-Sq Test was carried out to test for variance homogeneity.

	Chi-Sq.	df.	Probability
Peritoneal cavity	4.91	5	> 0.25
Liver	3.93	5	> 0.5
Total	4.38	5	> 0.5

As the variances are homogenous an ANOVA was carried out.

	DoF	SS	MS	Fraction	P
Peritoneal cavity					
Among group	5	680	136.1	1.0420	0.4135
Within group	27	3526	130.6		
Liver					
Among group	5	10302	2060	1.838	0.1390
Within group	27	30266	1120		
Total					
Among group	5	15727	3145	1.9349	0.1213
Within group	27	4389	1626		

The differences of none of the groups are significantly different at P = 0.05. However the 12 week serum does seem to give lower recoveries. Therefore a one tailed T-test using the 12 week serum results against the combined results of all the other groups was carried out.

	$\bar{x}$	DoF	SD	F test$^{*}_{(4.27)}$	$T_{(31)}$	P
12 week serum	47.8	4	25.41	0.334	-1.6376	0.059
All others	81.25	27	44.01			

* For homogeneity of variances

A one tailed T-test shows that the 12 week serum gives lower recoveries with a marginal significance of P = 0.059.

Experiment 6 Immunization of mice to tetrathyridia of M. corti

A. Using irradiated parasite

Groups	N	$\bar{x}$	S^2
i) a) Mice infected with irradiated parasite	7	3	32
b) Mice infected with irradiated parasite and challenged with normal parasite	7	110	4902
ii) c) Mice infected with irradiated parasite and challenged with normal parasite	5	63	981
d) Mice challenged with normal parasite	4	161	1722

On carrying out an F test, groups (c) and (d) have equal variances ($P > 0.05$).
A Student's T test was carried out to compare tetrathyridial multiplication between groups (c) and (d).

df	T	Significance
7	4.05	$P < 0.01$

The difference between the means of tetrathyridia recovered in groups (c) and (d) are significantly different showing that a primary infection with irradiated parasite protects the mouse against a secondary infection. A similar result was also obtained from a comparison between groups (a) and (b).

B. Using BCG

Groups	N	$\bar{x}$	S^2
a) Mice treated with BCG	6	274	7260
b) Control mice receiving saline	6	327	29743

On carrying out an F test, P is greater than 0.5% and therefore the 2 groups have equal variances. A student's T test was carried out to show whether treating the mice with BCG immunizes them against a tetrathyridial infection.

df	T	Significance
10	0.64	P > 0.05

This shows that injection of mice with BCG does not protect them against a tetrathyridial infection.

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